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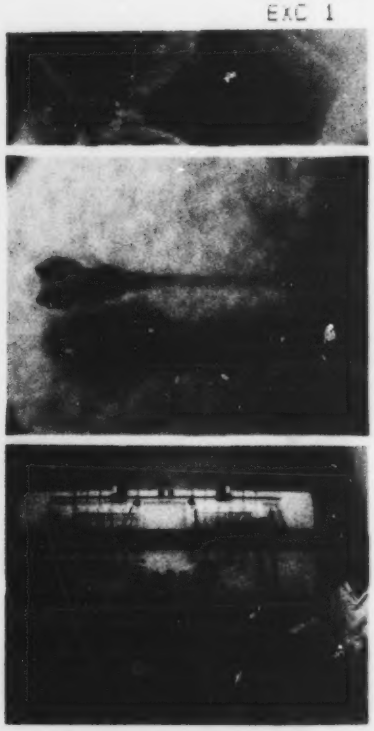

Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants

Report EPS 1/RM/12
August 1990

Canada

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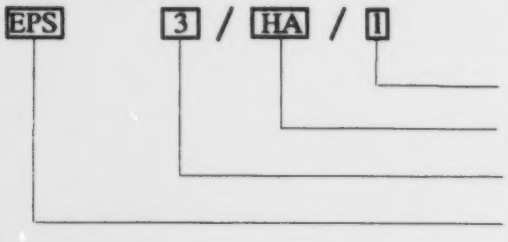

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Abstract

A reference toxicant is a chemical used in toxicity tests to provide results that can be compared within a laboratory or among laboratories. This document describes the use of reference toxicants within a laboratory for control of toxicity test precision over time. Thirteen chemicals were evaluated for their suitability as reference toxicants and the following meet specific criteria and have proven valuable in the respective toxicity tests.

Test Type	***Suitable Reference Toxicants***
96-hour rainbow trout lethality	*4-chlorophenol, phenol, sodium pentachlorophenate, hexavalent chromium, copper, zinc*
7-day fathead minnow survival and growth	*sodium pentachlorophenate, hexavalent chromium, copper, zinc*
48-hour Daphnia *spp. lethality*	*4-chlorophenol, phenol, sodium pentachlorophenate, hexavalent chromium, zinc*
3-brood Ceriodaphnia dubia *survival and reproduction*	*sodium pentachlorophenate, sodium chloride, hexavalent chromium*
96-hour Selenastrum capricornutum *growth inhibition*	*phenol, hexavalent chromium, copper, zinc*
96-hour threespine stickleback lethality	*4-chlorophenol, phenol, sodium pentachlorophenate, hexavalent chromium, zinc*
Microtox ™	*phenol, sodium pentachlorophenate*

Procedures for chemical acquisition, safe handling and storage are presented. It is recommended that testing be conducted at least once per month. Instructions are provided for establishing and interpreting control charts, as well as reporting data. Monitoring and interpreting duplicate test data are also discussed. Reference toxicant testing should be only one component of a continuous quality assurance/quality control program in aquatic toxicity testing laboratories.

Résumé

Un produit toxique de référence est un produit chimique utilisé dans les essais de toxicité pour produire des résultats intralaboratoires ou interlaboratoires comparables. Le présent document décrit l'utilisation de produits toxiques de référence en laboratoire pour le contrôle, dans le temps, de la precision des essais de toxicité. On a évalué treize produits chimiques afin de savoir s'ils pouvaient servir de produits toxiques de référence; les produits suivant respectent des critères précis et se sont avérés utiles dans les essais de toxicité dans le cadre desquels ils ont été utilisés.

Type d'essai	***Produits toxiques de référence appropriés***
Létalité à 96 heures chez la truite arc-en ciel	*4-chlorophénol, phénol, pentachlorophénate de sodium, chrome hexavalent, cuivre, zinc*
Survie et croissance à 7 jours de la tête-de-boule	*pentachlorophénate de sodium, chrome hexavalent, cuivre, zinc*
Letalité à 48 heures chez Daphnia *sp.*	*4-chlorophénol, phénol, pentachlorophénate de sodium, chrome hexavalent, zinc*
Survie et reproduction de la Ceriodaphnia dubia *à trois couvées*	*pentachlorophénate de sodium, chlorure de sodium, chrome hexavalent*
Inhibition de la croissance à 96 heures du Selenastrum capricornutum	*phénol, chrome hexavalent, cuivre, zinc*
Léthalité à 96 heures chez l'épinoche à trois épines	*4-chlorophénol, phénol, pentachlorophénate de sodium, chrome hexavalent, zinc*
Microtox ™	*phénol, pentachlorophénate de sodium,*

Le présent document présente les méthodes d'acquisition, de manutention sécuritaire et d'entreposage des produits chimiques. Il est recommandé d'effectuer les essais au moins une fois par mois. Des instructions sont énoncées pour la création et l'interprétation de tableaux de contrôle et pour le compte rendu des données. Ce document porte également sur la surveillance et l'interprétation des données de contre-essais. Les essais de produits toxiques de rérérence ne devraient constituer qu'un aspect d'un programme continu d'assurance et de contrôle de la qualité dans les laboratoires d'essais de toxicité aquatique.

Table of Contents

Abstract v

Résumé . . . . . *vi*

List of Figures . . . . . *ix*

List of Tables . . . . . *x*

Acknowledgements . . . . . *xii*

Section 1
Introduction 1
1.1 Background 1
1.2 Use of Reference Toxicants 1
1.3 Report Preparation Approach 2

Section 2
Reference Toxicant Selection 3
2.1 Initial Selection 3
2.2 Test-Specific Evaluation 5
2.3 Organic Chemicals 5
2.3.1 4-Chlorophenol 5
2.3.2 Dodecyl Sodium Sulphate 5
2.3.3 Phenol 7
2.3.4 Sodium Pentachlorophenate 7
2.4 Inorganic Chemicals 8
2.4.1 Cadmium 8
2.4.2 Chromium 9
2.4.3 Copper 10
2.4.4 Potassium Chloride 12
2.4.5 Silver Nitrate 12
2.4.6 Sodium Chloride 12
2.4.7 Zinc 13
2.5 Reference Toxicants in Sediment Tests 13
2.6 Use of Two Versus One Reference Toxicant 15

Section 3
Chemical Acquisition and Handling 17

Section 4
Conducting Tests 19
4.1 Testing Frequency 19
4.2 Chemical Confirmation of Test Solutions 19

Section 5
Warning Charts 21
5.1 Establishing and Updating Warning Charts 21
5.2 Data Interpretation 23
5.2.1 Warning Limits 23
5.2.2 Confidence Limits 24
5.2.3 Data Trends 24
5.2.4 Training New Technicians 25

Section 6
Duplicate Testing 26
6.1 High vs. Low Duplicate Plots 26
6.2 Range-Type Control Charts for Effluents 28
6.3 Range-Type Control Charts for Reference Toxicants . . 28

Section 7
Record - keeping and Data Reporting 29

References 31

Appendix A
Reference Toxicant Selection 39

Appendix B
Health and Safety Data 67

Appendix C
Individuals Who Provided Information on Reference Toxicant Evaluation 79

Appendix D
Literature Sources 81

Appendix E
Test Solution Preparation 83

List of Figures

1 Empirical Relationship Between Total Hardness of Fresh Water and Toxicity of Copper to Aquatic Organisms 11

2 Empirical Relationship Between Total Hardness of Fresh Water and Toxicity of Zinc to Aquatic Organisms 14

3 Mean Chart 22

4 High-Low Duplicate Control Chart 27

5 Range-Type Control Chart 27

List of Tables

1 Ranking of Potential Reference Toxicants Acccording to Primary Selection Criteria 4

2 Suitability of Various Reference Toxicants in Specific Toxicity Tests 6

3 Chemical Suppliers 18

A.1 Ranking of Potential Reference Toxicants According to Primary Selection Criteria 51

A.2 4-Chlorophenol (*para*-Chlorophenol) Supporting Documentation 52

A.3 Dodecyl Sodium Sulphate (DSS) Supporting Documentation 53

A.4 Phenol Supporting Documentation 54

A.5 Sodium Pentachlorophenol(NaPCP) Supporting Documentation 55

A.6 Cadmium Chloride($CdCl_2$) Supporting Documentation 56

A.7 Hexavalent Chromium (as $K_2Cr_2O_7$ or $KCrO_4$) Supporting Documentation 57

A.8 Copper Sulphate ($CuSO_4$) Supporting Documentation 58

A.9 Potassium Chloride (KCl) Supporting Documentation 59

A.10 Silver Nitrate ($AgNO_3$) Supporting Documentation 60

A.11 Sodium Chloride (NaCl) Supporting Documentation 61

A.12 Zinc Sulphate($ZnSO_4$ or $ZnCl_2$) Supporting Documentation 62

A.13 Reproducibility of Results Using Specific Reference Toxicants in Specific Test Types 63

A.14 Acute Toxicity of Copper to Fish 63

A.15 Chronic Toxicity of Copper to Fish 64

A.16 Acute Toxicity of Copper to Invertebrates 65

A.17 Chronic and Sublethal Toxicity of Copper to Invertebrates 65

A.18 Acute Toxicity of Zinc to Fish 66

B.1 4-Chlorophenol Health and Safety Data 68

B.2 Phenol Health and Safety Data 69

B.3 Sodium Pentachlorophenate Health and safety Data 70

B.4 Cadmium Chloride Health and Safety Data 71

B.5 Potassium Dichromate Health and Safety Data 72

B.6 Potassium Chromate Health and Safety Data 73

B.7 Cupric Sulphate Health and Safety Data 74

B.8 Potassium Chloride Health and Safety Data 75

B.9 Sodium Chloride Health and Safety Data 76

B.10 Zinc Sulphate Heptahydrate Health and Safety Data 77

E.1 Examples of Test Solutions' Preparation Steps 85

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Section 1

Introduction

1.1 Background

New aquatic toxicity testing protocols are being developed by Environment Canada to be used in Canadian laboratories for the assessment and control of individual substances and substances present in liquid industrial or municipal facility wastes, groundwater leachates and surface waters. Laboratories will differ with respect to dilution water quality, genetic history of test organisms, technician training and experience, etc., which may lead to varying test results.

It will be important to Environment Canada and to industry that quality assurance/quality control (QA/QC) programs be implemented in each laboratory to ensure that comparable results can be achieved by different laboratories. Among other components of laboratory QA (Hart, 1990), laboratories will be required to conduct reference toxicant tests.

1.2 Use of Reference Toxicants

A reference toxicant is simply a chemical that is used in toxicity testing to make comparisons between test results.

Reference toxicants have been used in interlaboratory testing to judge comparability of results between laboratories. Toxicity test accuracy can be inferred through round-robin testing, where the consensus among endpoints of the majority of laboratories is assumed to reflect the 'true' result. However, the concept of a 'true' result as it is applied in chemical analyses is inappropriate to the interpretation of interlaboratory biological tests. Reasons for this include diffences in dilution waters and organism stocks, and the fact that some test elements are not precisely defined (e.g., rate of aeration), which can lead to different, but equally valid, test results.

A second common function of reference toxicants is to provide a general measure of the reproducibility (precision) of a toxicity test method within a single laboratory over time. Individual results are compared with historical test performances to identify whether they fall within an acceptable range of of variability. Data that fall outside of established limits trigger a review of potential sources of variability. Variability may be attributed to factors such as test organism health, differences among batches of organisms in genetic tolerance to toxicants, changes in laboratory water quality, and the operational consistency of technicians.

Since the two functions of reference toxicants address two separate components of laboratory QA, the necessary characteristics of the substances used for each function differ. Some, but not all, reference toxicants will share the characteristics required for both purposes. This document addresses only the use of reference toxicants in the control of toxicity test precision.

1.3 Report Preparation Approach

The objective of this project was to develop an instruction manual to be used by aquatic toxicity laboratories in the control of toxicity test precision.

A computerized search (Appendix D) identified literature on the use of various chemicals as reference toxicants. Review documents for each chemical were also consulted. Each chemical was evaluated in terms of characteristics determined to be important in monitoring toxicity test precision (Section 2 and Appendix A). Then each chemical was evaluated for suitability with respect to specific types of toxicity tests.

Information on the acquisition and safe handling of the chemicals has been provided (Section 3, Appendix B). Instructions regarding data analysis and interpretation (Sections 5 to 7) were adapted from U.S. Environmental Protection Agency (U.S. EPA) methods (Weber *et al.*, 1989), and procedures used in chemical analytical laboratories (Dux, 1986; King, 1984).

Section 2

Reference Toxicant Selection

2.1 Initial Selection

Four organic (4-chlorophenol, dodecyl sodium sulphate, sodium pentachlorophenate, and phenol) and nine inorganic (cadmium chloride, copper sulphate, potassium dichromate, potassium chromate, potassium chloride, sodium chloride, silver nitrate, zinc chloride and zinc sulphate) chemicals were evaluated to determine their suitability as reference toxicants. The evaluation process involved two stages (Appendix A). Initially each chemical was scored according to eight criteria (Table 1).

While the rationale behind each selection criterion is generally self-evident, two require discussion. The first pertains to the ability of reference toxicant tests to detect abnormal organisms. This has often been cited or assumed to be an important, if not primary, characteristic of reference toxicants. It was evident from the literature search, and through conversation, that alarmingly little research has been conducted to verify that any reference toxicants are actually able to consistently reflect poor organism health or genetically different stocks. This function of reference toxicants was still identified as important in this project, but until more data become available regarding the relative extent to which all sources of test variability affect test results, the objective of reference toxicant testing has been defined as a general measure of test reproducibility in a single laboratory over time.

The second selection criterion that requires clarification is the effect of variations in laboratory water quality on toxicity test results. Two alternatives arose:

- the toxicity of the reference toxicant should be highly sensitive to water quality in order to also monitor the extent to which this source of variability could affect other test results; and
- the toxicity of the reference toxicant should not be sensitive to normal water quality changes within a single laboratory in order that the effects on toxicity test results of other sources of variability can be isolated (e.g., organism health, technician performance).

While advantages and disadvantages were identified for each approach, the first was ruled out because no single toxicant will give toxicity test results that reflect all water quality changes, nor could the degree of response be extrapolated to other test solutions (e.g., effluents). Contaminants were, therefore, given a positive score on this criterion (Table 1) if relatively consistent test results were achievable in a given laboratory over time (presumably despite limited water quality variations). It was recognized, however, that the toxicity of a few contaminants is unaffected by water quality changes. It was also assumed that some laboratories may occasionally experience relatively large changes in some water quality parameter(s). For this reason, some discussion of water quality effects on

Table 1 Ranking[a] of Potential Reference Toxicants According to Primary Selection Criteria[b]

	Organic				Inorganic						
Criteria[c]	4-Chloro phenol	Dodecyl Sodium Sulphate (DSS)	Phenol	Sodium Pentachloro-phenate (NaPCP)	Cadmium ($CdCl_2$)	Chromium ($KCrO_4$) or ($K_2Cr_2O_7$)	Copper ($CuSO_4$)	Potassium Chloride (KCl)	Silver Nitrate ($AgNO_3$)	Sodium Chloride (NaCl)	Zinc ($ZnSO_4$)
Detection of Abnormal Organisms	0	no	yes	yes	0	E	no	0	0	E	yes
Established Toxicity Database	no	yes	yes	yes	yes	yes	yes	no	yes	yes	yes
Readily Available in Pure Form	yes	yes[g]	yes	yes	yes	yes	yes	yes	yes	yes	yes
Soluble	yes	yes[d]	yes	yes	yes	yes	yes[d]	yes	yes	yes	yes
Stable in Solution	yes	no	no	yes	yes	yes	yes[d]	yes	E	yes	yes
Stable Shelf Life	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Limited Intralaboratory Water Quality Effects	yes	yes	yes	E[e]	0[f]	yes[e,f]	yes[d,f]	yes	no	yes	yes[f]
Easily Analyzed	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
TOTAL SCORE	5	4	6	7	6	7	6	5	4	7	8

E = equivocal; conflicting reports
0 = too few data

[a] Addition of "yes" items and subtraction of "no" gives total; other symbols (0 and E) have no score
[b] Supporting data presented in Appendix Tables A.2 to A.12
[c] Complete description in text, Appendix A
[d] Not in some waters
[e] Some pH effects
[f] Some hardness effects
[g] Batches may vary in toxicity

toxicity has been included in the following sections to aid laboratories in reference toxicant selection (e.g., not to select a reference toxicant whose toxicity is sensitive to a water quality parameter that is inconsistent in that laboratory) and in determining whether unusual reference toxicant test results can be attributed to changes in a particular water quality parameter.

Chemicals that scored five or more out of a possible eight in the initial selection process were further evaluated for their suitability in specific test types (Table 2).

2.2 *Test-Specific Evaluation*

Few data were available in the literature (Appendix D) relating specifically to the use of reference toxicants in the control of test precision (Appendix A). A number of experienced North American scientists were contacted and asked to provide additional data and/or insights. In the following pages, references to unpublished data (as opposed to personal communications) pertain to data that was physically provided to the authors. Source information for personal communication references is provided in Appendix C.

The information presented in Table 2 is intended only as a guide, since some judgements were made on limited information. Some chemicals for which no data were available may prove to be good reference toxicants after further testing. Also, some chemicals have appeared to work well in some laboratories, but not in others, although the reason was not always clear (some explanations are presented in the text; possible factors include laboratory water quality, genetic tolerance of organisms, etc.). If unsatisfactory results can be attributed to any of the factors that a reference toxicant is intended to monitor (e.g., organism health, competence of technical staff), laboratories are encouraged to try another chemical.

2.3 *Organic Chemicals*

2.3.1 4-Chlorophenol

The use of 4-chlorophenol as a reference toxicant is being investigated at the aquatic toxicity laboratories of the Ontario Ministry of Environment in Rexdale, Ontario, and Environment Canada in Dartmouth, Nova Scotia. Preliminary acute lethality tests using rainbow trout, threespine stickleback (*Gasterosteus aculeatus*) and *Daphnia magna* have demonstrated adequate reproducibility (D. Poirier, unpublished data; K. Doe, unpublished data), although in six of seven acute *Daphnia magna* tests conducted at the latter laboratory, a poor dose response relationship was observed (K. Doe, unpublished data). Very little additional toxicity data for this chemical are available in the literature. Unlike phenol (below), 4-chlorophenol appears to be stable in solution (K. Doe, unpublished data). In crystalline form, the chemical emits a strong odour (Keith and Walters, 1985) and adequate ventilation should be ensured (Appendix B).

2.3.2 Dodecyl Sodium Sulphate (DSS)

Dodecyl sodium sulphate is not recommended as a reference toxicant. Rainbow trout stressed by starvation, temperature, or crowding did not respond (median survival time) differently to DSS than "normal" fish, nor were different strains of trout distinguishable (Alexander and Clarke, 1978). Pessah *et al.*, (1975) also reported that the acute LC_{50} for a diseased stock of rainbow trout was not different from that for healthy fish. DSS also degrades in solution (Pessah *et al.*, 1975; Abel, 1974) and different batches of

Table 2 Suitability of Various Reference Toxicants in Specific Toxicity Tests*

	Organic			Inorganic				
Test Type	4-Chloro-phenol	Phenol	Sodium Penta-chlorophenate (NaPCP)	Cadmium ($CdCl_2$)	Hexavalent Chromium (as $K_2Cr_2O_7$ or K_2CrO_4)	Copper ($CuSO_4$)	Sodium Chloride (NaCl)	Zinc $ZnSO_4$ or $ZnCl_2$)
96-hour rainbow trout lethality	yes	yes	yes	no	yes	yes	no	yes
7-day fathead minnow larval survival and growth	?	?	yes	no	yes	yes	?	yes
48-hour *Daphnia* sp. lethality	yes	yes	yes	no	yes	?	?	yes
3-brood *Ceriodaphnia dubia* survival and reproduction	?	?	yes	?	yes	?	yes	?
96-hour *Selenastrum capricornutum* growth inhibition	?	yes	?	no	yes	yes	?	yes
96-hour threespine stickleback lethality	yes	yes	yes	no	yes	no	no	yes
Microtox™	?	yes	yes	no	?	no	no	no

* Supporting data presented in Appendix A.

? = No data available.

chemical could demonstrate different toxicities (Fogels and Sprague, 1977; K. Doe, unpublished data).

2.3.3 Phenol

Phenol tests showed differences in sensitivity among strains of trout and could discern the effects of starvation, temperature stress, and pre-exposure to 40 µg/L chlorine on the sensitivity of trout to phenol, but not the effects of three brands of food or high mortality during holding (Alexander and Clarke, 1978). Another laboratory reported that a nutritional deficiency in a rainbow trout stock became evident when phenol was used as a reference toxicant (Somers, unpublished data).

The effect of microbial degradation on laboratory toxicity tests using phenol has not been reported in the literature, but one laboratory (K. Doe, unpublished data) demonstrated greater than 90 per cent phenol removal in test solutions (initial nominal concentrations of 10 and 18 mg/L) at the completion of a rainbow trout bioassay (96 hours). Rapid reductions in aquatic phenol concentrations have been observed by others (Brown *et al.*, 1967; Westlake, personal communication, as cited by Lee, 1980). Photodegradation (Buikema *et al.*, 1979) and volatilization (Lee, 1980) have been proposed as potential mechanisms of phenol removal from solution. Doe (unpublished data), however, demonstrated that phenol concentrations remained stable during four days of aeration and exposure to typical laboratory lighting conditions prior to the introduction of test trout. The U.S. EPA Treatability Manual (1983) also indicates that microbial degradation is a more important removal process for aquatic phenol than volatilization or photodegradation. This would suggest that bacteria associated with the trout were responsible for phenol removal during the trout bioassay.

It was suggested that dissolved oxygen levels could decline to unacceptable levels in unaerated tests (D. Poirier, personal communication), presumably as a result of microbial activity, but no data were found (or provided) to support this concern. In fact, reproducible results have also been achieved in static invertebrate and algae tests (Appendix A).

Phenol might not give consistent results in dilution waters that contain bacteria since degradation could begin as soon as solutions are prepared. Fresh test solutions must be prepared for each test.

Neither water hardness nor pH would be expected to have a substantial effect on phenol toxicity over the range of values expected for those parameters in most laboratory waters (Buikema *et al.*, 1979).

2.3.4 Sodium Pentachlorophenate (NaPCP)

Rainbow trout stressed by crowding or starvation showed a difference in median survival time from unstressed fish. Temperature-stressed fish showed no difference nor was any difference detected between genetic strains. Of five toxicants tested, phenol was slightly better than others at detecting differences between groups of trout (sodium azide, copper sulphate, dodecyl sodium sulphate also tested) (Alexander and Clarke, 1978).

The toxicity of PCP is pH-dependent with toxicity increasing at lower pHs (Dave, 1984; Lee, 1980; Kobayashi and Kishino, 1980). DeGraeve *et al.* (1989) reported that NaPCP toxicity was sufficiently pH-related that pH shifts in exposure vessels during tests could cause substantial variability between chronic toxicity tests to fathead

minnow larvae. The pKa for pentachlorophenate is approximately 5 (Buikema *et al.*, 1979) and, at pH 7.4 or greater, PCP is 99 per cent ionized (Alderdice, 1963). If PCP toxicity was strictly associated with the unionized chemical form (Kobayashi and Kishino, 1980; Dave, 1984; Lee, 1980), bioassays conducted at pH above 7.4 should show relatively consistent results. In fact, 24-hour goldfish LC_{50}s generated at pH 9 were an order of magnitude greater than those at pH 8 (Kobayashi and Kishino, 1980). Conversely, LC_{50}s generated at pH 5.5 and 7 differed by a factor of only 1.6. This suggests that the pKa may actually fall above 7, and/or that other factors are involved in PCP toxicity besides the proportion of ionization.

Despite the above, NaPCP reference toxicant tests conducted with fish and invertebrates in the laboratory of Beak Consultants Ltd. have given consistent results (coefficients of variation of 14 to 30 per cent over all test endpoints, dilution water pH of 7.6 to 8.4) (BEAK, unpublished data, Appendix A). Sodium pentachlorophenate has also been recommended as a good reference toxicant by others (Lee, 1980; Chapman, 1988).

NaPCP is highly toxic and is carcinogenic (Sax and Lewis, 1987). Laboratories that choose to use NaPCP as a reference toxicant should follow appropriate handling procedures (Appendix B).

2.4 Inorganic Chemicals

2.4.1 Cadmium (as $CdCl_2$)

Dissolved cadmium in fresh waters consists of free metal ions, weak complexes and colloids (all labile), and inert complexes (non-labile). The free ion is typically predominant, although speciation can be influenced by hardness, pH, redox potential, suspended particulate and organic matter (McCracken, 1987; Sprague, 1987). Soluble complexes are formed with chlorides and sulphate, while precipitates can occur with carbonate,and hydroxide and sulphide ions. Clays, muds, humic and organic material, and some hydrous oxides may strongly adsorb cadmium (U.S. EPA, 1980a).

Since the labile cadmium species are more readily bioavailable, they are the most toxic (McCracken, 1987). Toxicity of cadmium to freshwater salmonids is particularly related to water hardness, which governs the speciation of cadmium (e.g., $CdCO_3$ or $Cd(OH_2)$ precipitates will form at high hardness) and also affects biological factors in the fish that determine the rates of cadmium uptake; elevated calcium levels and , to a lesser extent, magnesium in high hardness waters appear to reduce gill permeability to cadmium (Sprague, 1987). In seawater, toxicity is inversely related to salinity because of the complexation of free cadmium ions by chloride ions (Sunda *et al.*, 1978).

Acute exposures of fish and invertebrates to cadmium can result in narcotization which could lead to misinterpretation of immobile versus dead organisms (Lamberson and Swartz, 1990; B. Peltier, pers. comm.).

The toxicity curve for salmonids (cadmium concentration versus median survival time) shows a typical relationship over the first one to six days of exposure, but after that survival time does not change over two orders of magnitude of cadmium concentration. This indicates a secondary mechanism of toxicity that is characterized by violent and uncoordinated activity on external stimulus (Sprague, 1987). Disturbances of test organisms that occur in the course of usual monitoring procedures

have been shown to reduce the acute LC_{50} (Benoit *et al.*, 1976). This secondary mechanism of toxicity appears also to exist for fathead minnows and sticklebacks, but not other species (Sprague, 1987).

Thresholds of acute lethality to salmonids can be expected at about 2.5 µg Cd/L in very soft water, ranging up to 27 µg/L in very hard water. Lethal concentrations for other freshwater species are widely scattered around a geometric average of about 1660 µg/L with no apparent relation to water hardness (Sprague, 1987). Most adult and juvenile marine fish are not particularly sensitive to cadmium; acute LC_{50}s are generally greater than 10 000 µg/L. Early life stages are typically unaffected at concentrations below 1000 µg/L (McLeese *et al.*, 1987). Larval marine and freshwater invertebrate stages are toxically affected in the low microgram per litre range, but later life stages (except Cladocerans) can tolerate a few hundred to a few thousand micrograms of cadmium per litre (McLeese *et al.*, 1987; Wong, 1987). Algae are generally not affected above 50 µg/L (McLeese *et al.*, 1987), except *Selenastrum capricornutum* which gives a 96-hour EC_{50} of 6 to 17 µg/L (Wong, 1987; U.S. EPA, 1989b).

It is possible that the unusual toxic action of cadmium on trout, fathead minnows, and sticklebacks will introduce unacceptable variability between tests; limited data indicate poor repeatability (Appendix A, Section A.2.2.1). Although Cladocerans and algae are quite sensitive to cadmium toxicity, limited testing of those organisms has also given poor results. Cadmium is also not recommended for use in Microtox™ testing since bacteria generally tolerate cadmium well.

No information was found regarding whether cadmium chloride toxicity tests will distinguish between normal and abnormal groups of organisms.

In addition, cadmium is carcinogenic (Sax and Lewis, 1987). This chemical does not appear to be a good choice as a reference toxicant for most toxicity tests.

2.4.2 Chromium ($K_2Cr_2O_7$ or K_2CrO_4)

Chromium salts distribute themselves into three predominant species according to the following equilibria (Jop *et al.*, 1987):

$$Cr_2O_7^{-2} + H_2O \longrightarrow 2HCrO_4^- \quad (1)$$

$$2HCrO_4^- \rightleftharpoons 2CrO_4^{-2} + 2H^+ \quad (2)$$

Equation 1 indicates that most aqueous dichromate rapidly combines with water to form hydrochromate ($HCrO_4^-$). Equation 2 is pH-dependent with the equilibrium increasingly tending to the right at pH greater than 6.5. Conversely, at pH less than 6.5, the equilibrium shifts to the left (Jop *et al.*, 1987). Since $HCrO_4^-$ is more readily taken up by aquatic species than the other chromate species (Jop *et al.*, 1986), it follows (from Equation 2) that the toxicity of a given quantity of hexavalent chromium (Cr^{6+}) would be enhanced by lower ambient pH.

In a toxicity test, the introduction of dichromate into an aqueous solution of basic pH would result in the generation of hydrogen ions (Equation 2). This would lead to a measurable drop in pH at sufficiently high chromium concentrations and in poorly buffered waters, such that the equilibrium between chromium species would, again, shift. More $HCrO_4^-$ would then be formed (due to lower pH) and the toxicity of the solution would be enhanced. Since the extent of this reaction would be

chromium concentration-dependent, different equilibria would result in different test dilutions.

Conversely, addition of the chromate salt to solutions with a pH of 7.0 or more would be unlikely to produce a measurable pH change.

Jop *et al.* (1987) demonstrated the above points by testing the relative toxicity of chromate and dichromate to *Daphnia pulex*, *Mysidopsis bahia*, *Cyprinodon variegatus*, *Pimephales promelas*, *Gasterosteus aculeatus*, and *Lepomis macrochirus*. The dilution waters used (marine and fresh water) had a pH of 7.5 or greater. The invertebrates showed greater sensitivity to Cr^{6+} (LC_{50}s of 0.18 to 6.3 mg/L) relative to fish (LC_{50}s of 38 to 214 mg/L). The authors observed that the toxic concentrations of dichromate tested in invertebrate tests were not sufficient to produce obvious changes in test solution pHs and, as a result, the relative toxicities of chromate and dichromate were similar.

In the fish tests, however, the highest test concentrations showed different rates of mortality between fish tested using the two chemicals (particularly initially), and for one species (*P. promelas*) the chromate and dichromate salts produced significantly different LC_{50}s. The authors concluded that using a chromate salt would probably minimize potential variability due to pH changes at different exposure concentrations. This would be true, however, only under alkaline pH conditions. With a pH less than 6.0 (acidic conditions), it would be desirable to use the dichromate salt since chromate at such pHs would result in a reduction of free aqueous hydrogen ions (Equation 2) and, therefore, an increase in pH. In dilution waters of pH 6.5 , the pKa of dichromate, the relative proportion of $HCrO_4^-$ and CrO_4^{-2} would be 50:50. It would appear that even the minor pH differences that can occur with the addition of either chromate or dichromate near the pKa value could substantially affect the speciation and, therefore, toxicity of the solution.

Even in view of these results, Jop *et al.* (1987) concluded that hexavalent chromium toxicity is probably not highly pH-dependent under laboratory conditions. To minimize pH effects, however, it is recommended that laboratories with dilution water pH of 7.0 or more use the chromate salt if chromium is selected as the reference toxicant. Laboratories with dilution water pH of 6.0 or less should probably use dichromate. Laboratories with water supplies of pH between 6.0 and 7.0 may want to choose an alternative toxicant, or to experimentally verify that pH effects do not cause an unacceptable degree of variability between tests.

Chromium has given reproducible results in most toxicity tests (see Appendix A); however, it is carcinogenic, and easily absorbed through the skin (Sax and Lewis, 1987). Laboratory staff should take proper precautions when handling this chemical (Appendix B).

2.4.3 Copper (as $CuSO_4$)

Aqueous copper can exist in many forms, with each exhibiting varying degrees of toxicity (Spear and Pierce, 1979). The cupric (Cu^{2+}) ion is highly reactive, forming moderate to strong complexes and precipitates with inorganic and organic constituents of natural waters (e.g., carbonate, phosphate, amino acids, humates, suspended solids) (U.S. EPA, 1980d). Alkalinity and pH govern copper speciation in the absence of other complexing or adsorbing agents by formation of carbonate and hydroxy

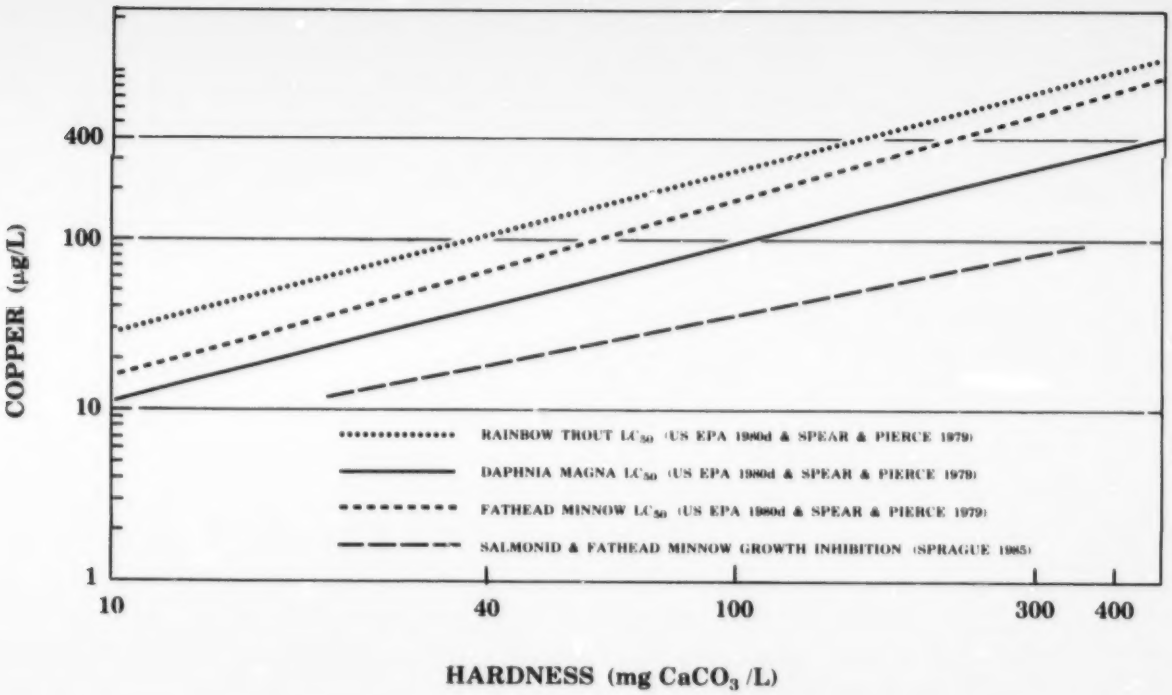

Figure 1 **Empirical Relationship Between Total Hardness of Fresh Water and Toxicity of Copper to Aquatic Organisms**

complexes. In marine waters, the degree of inorganic complexation tends to increase with increases in salinity. Colloidal dispersions of hydroxy and carbonate species can also be abundant in seawater (Spear and Pierce, 1979).

Generally, the formation of strong ligands or colloids, or adsorption to particulate matter, reduces the aquatic toxicity of copper (Spear and Pierce, 1979; U.S. EPA, 1980d). Laboratories using dilution water that could contain variable levels of complexing materials (e.g., untreated surface waters) are not advised to select copper as a reference toxicant, since unacceptable variability could occur in test results. This applies particularly to natural seawater supplies.

The relation between water hardness and toxicity differs between organisms and some examples are approximately represented by the lines on Figure 1. In waters that do not contain high levels of complexing materials, and where hardness levels remain relatively consistent, acute toxicity tests using fish typically give reproducible results (Appendix A, Table A.14). Growth is a sensitive indicator of copper toxicity to fish (Spear and Pierce, 1979); therefore copper is an appropriate reference toxicant for chronic tests that use growth as an end point. Consistent results have been obtained in some acute and chronic invertebrate tests and algae tests, but variable water quality (pH, hardness) could cause inconsistent results. Marine tests using natural water for dilution could give poor results.

Alexander and Clarke (1978) reported that starved or crowded rainbow trout did not show significantly different responses to

copper (as median survival time) than unstressed fish, nor were different genetic strains distinguishable. This contrasted with the phenol or NaPCP tests (previously mentioned) which did show differences in sensitivity between different groups.

If improperly stored, copper sulphate can absorb water. Precautions should be taken to ensure that the container is well-sealed, and preferably stored in a dessicator (Appendix B).

2.4.4 Potassium Chloride (KCl)

Potassium chloride is being evaluated by the U.S. EPA for its suitability as a reference toxicant and preliminary tests have given encouraging results (B. Peltier, G. Callous, pers. comm.). Potassium chloride may be superior to other toxicants because water quality has little or no effect on its toxicity. Unlike sodium chloride, it would be suitable in marine tests. It is less toxic to humans and therefore easier to handle in the laboratory than many other reference toxicants. No test data have yet been made available; therefore, it cannot be recommended as a reference toxicant at this time.

2.4.5 Silver Nitrate ($AgNO_3$)

Silver nitrate is not recommended as a reference toxicant. Very little information was obtained to show whether reproducibility could be achieved in repeated tests, since the chemical has seldom been used as a reference toxicant. Limited data indicated that the toxicity of silver nitrate is highly hardness-and salinity-dependent (Lemke, 1981; Dinnel *et al.*, 1987), suggesting that even water quality variations anticipated within a single laboratory could cause inconsistent toxicity results. Silver nitrate was also unstable in heavily-aerated solutions (Lemke, 1981). Considering also that it is a toxic, highly reactive chemical, and therefore difficult to handle in the laboratory it was clear that other candidate chemicals would be more suitable for reference toxicant testing.

2.4.6 Sodium Chloride (NaCl)

A potential drawback to the use of NaCl in freshwater reference toxicant testing is that organisms could be too insensitive to NaCl for tests to detect abnormal test organisms. Diseased fish stocks have given "normal" acute LC_{50}s in two laboratories (T. Kovacs, personal communciation; Hansen *et al.*, 1979). On the other hand, one laboratory reported that goldfish from a stock displaying high holding mortality had an LC_{50} when exposed to sodium chloride that was out of the range found when healthy stocks were used (Adelman and Smith, 1976).

Other critical information to consider relates to the repeatability of tests using sodium chloride. Some laboratories that use NaCl as a reference toxicant report that test results "never" exceed the 95% confidence limits (confidence limits are discussed in Section 5). At the 95 per cent confidence level, at least five per cent of data can be expected to fall outside of the limits due to chance (Section 5), in addition to occasional tests in which mistakes or problems occur. Also, the coefficients of variation reported for most inter-and intralaboratory studies are frequently better than for any other toxicant (Appendix A). This indicates two contrasting possibilities: that sodium chloride is an excellent reference toxicant, or that sodium chloride tests are not sufficiently sensitive to indicate abnormal conditions. Further testing and/or publication of existing data is/are required to resolve this issue.

The greatest advantage of sodium chloride over other toxicants is that it is not toxic to

laboratory staff and is, therefore, safe to handle. It is not a suitable reference toxicant for marine tests.

2.4.7 Zinc ($ZnSO_4$)

Except in waters with high alkalinity and pH greater than 7.5, the aquo ion of zinc ($Zn(H_2O_6)^{2+}$) is likely the predominant and most toxic zinc species. The proportion of aquo ions decreases with increasing salinity, pH, and/or alkalinity, generally resulting in decreased toxicity to fish, invertebrates, and algae. Suspended molecules of zinc carbonate ($ZnCO_3$; formed at high pH or alkalinity) could also be quite toxic, but suspended zinc hydroxide ($Zn(OH)_2$) is relatively non-toxic (Spear, 1981).

In seawater, $ZnCl_2$ and $ZnCl^+$ increase as salinity increases, although the aquo ion can remain as the dominant species. Substantial amounts of sulphate and carbonate forms can also occur.

Zinc can also complex with organic materials, such as humic acids, in marine and freshwaters, although the stability of such complexes is typically low. In the presence of adsorbing agents and organic chelations of high molecular weight, zinc can co-precipitate, particularly as the pH increases above 6.0.

With respect to zinc toxicity to fish, two competing mechanisms appear to operate: as the pH rises, dissolved zinc becomes increasingly toxic, but at higher pH levels, it is increasingly replaced by zinc precipitate, which is of low toxicity to fish (Bradley and Sprague, 1985). A similar water quality versus toxicity relationship (at least for hardness) likely exists with other aquatic species (Figure 2).

For laboratories using dilution waters with relatively stable pH and hardness, zinc is likely a good reference toxicant choice for most tests; consistent results over time appear to be achievable and zinc represents a lesser human health hazard than some of the other chemicals.

2.5 Reference Toxicants in Sediment Tests

Sediments have been well-recognized as a sink or source of persistent toxic chemicals (Zarba, 1989; Ross and Henebry, 1989; Karickhoff and Morris, 1985). Sediment contaminants exhibit complex interactions with physical/chemical properties of the sediment (Karichoff *et al.*, 1979). Organic carbon content, for example, is directly related to contaminant sorption, particularly for hydrophobic chemicals. This reduces toxicity since the chemical is less available for uptake by biota. Similarly, contaminant sorption tends to decrease with increased particle size, resulting in increased bioavailability and toxicity (Karickhoff *et al.*, 1979).

Sediment toxicity tests have been developed to measure the biological impact of sediment-associated contaminants (e.g., Swartz *et al.*, 1985; Nebeker *et al.*, 1984; U.S. EPA/U.S. ACOE, 1977). A variety of approaches have been used in sediment assays depending on the objectives of the study, e.g.:

- spiking - contaminant(s) is/are mixed into sediment or added to overlying water; duration of mixing stage important;
- mixing - combination of different sediment layers;
- seiving - to achieve homogeneity of particle size;
- dilutions - to establish dose-response;

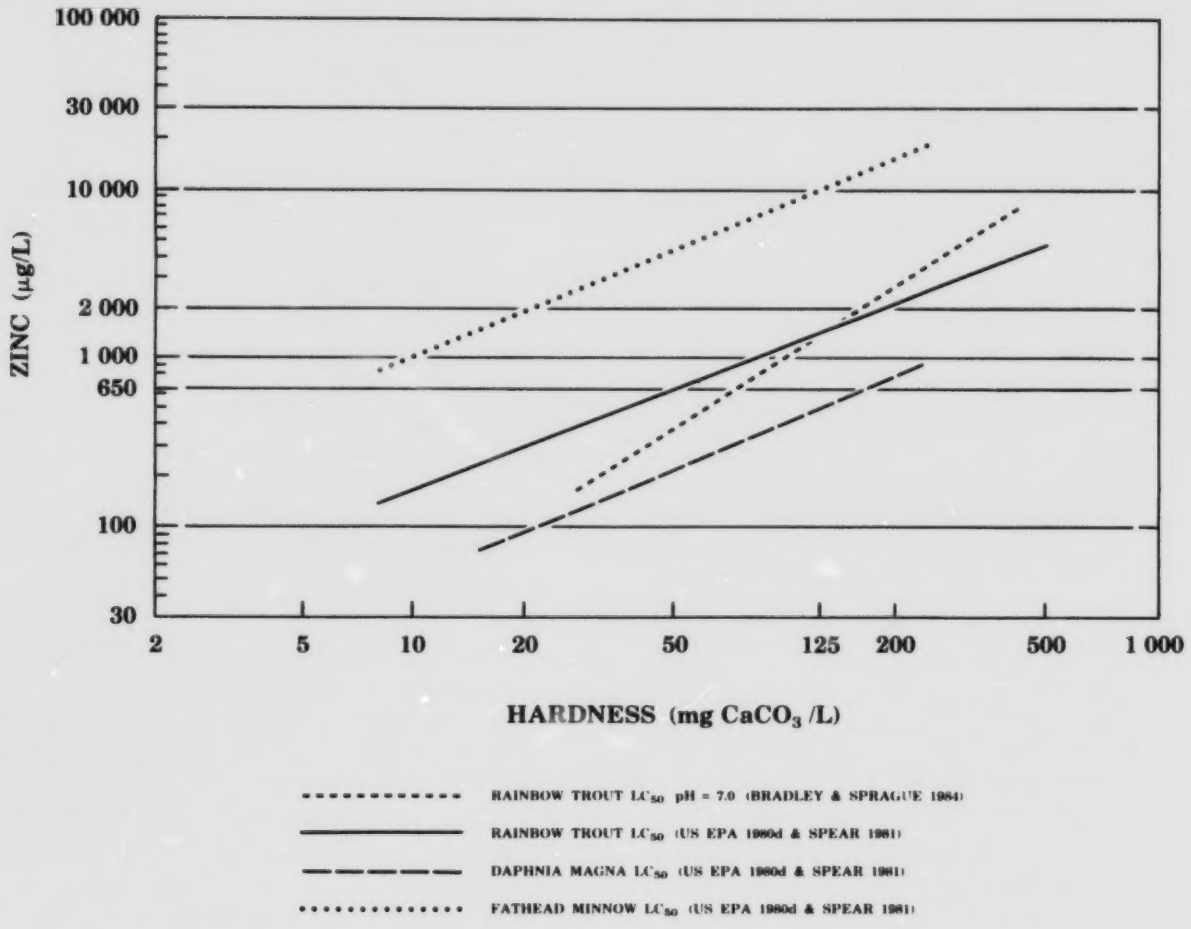

Figure 2 **Empirical Relationship Between Total Hardness of Fresh Water and Toxicity of Zinc to Aquatic Organisms**

- elutriation - determination of toxicity of water-soluble phase; and
- sterilization - inhibition of biological activity.

Some tests may combine more than one of the above approaches and may involve the use of either standardized or natural sediments.

Spiking control sediments with a reference toxicant to monitor test precision is conceptually analogous to using reference toxicants in aquatic toxicity tests using laboratory dilution water. In sediment testing, however, a control typically consists of a "clean" sediment (lacking the contaminants of the test sediment) that has characteristics similar to those of the test sediment (e.g., organic carbon content, particle size distribution). In this, sediment tests often differ from aquatic tests since

control medium characteristics differ between tests, whereas dilution water controls in aquatic tests are relatively consistent. Therefore, if different control sediments were spiked with a reference toxicant, it is unlikely that the sources of variability that the laboratory wishes to monitor and control (e.g., organism health, technician performance, etc.) could be separated from the variability associated with differences beween each test system (e.g., sediment characteristics).

To partially address this problem, waterborne reference toxicant exposures of sediment (benthic) test organisms have been recommended as a QC measure with sediment tests (Tetra Tech Inc. and E.V.S. Consultants Inc., 1986, Lamberson and Swartz, 1990). Test duration may need to be restricted (e.g., four rather than ten days) since biota could become stressed by lack of substrate (R. Swartz, personal communication), and water column tests are probably inappropriate for biota that are highly dependent on substrate (e.g., chironomids). While water column tests provide a measure of variability associated with some aspects of sediment tests (e.g., seasonal sensitivity of test organisms), they do not provide an overall measure of the reproducibility of a given sediment toxicity test method in a given laboratory (e.g., the objective identified in Section 1 for aquatic toxicity tests).

A suggested additional/alternative QC practice in water column tests is to use a standardized sediment spiked with reference toxicant to monitor test precision over time. The test method employed should be the same (or as representative as possible) as the method typically used in the laboratory. Separate reference toxicant tests should be performed for each distinct test method/test species combination. The sediment spiking method should be well-defined to reduce the error associated with contaminant bioavailability. It is critical to system control that the characteristics of the standardized sediment remain consistent over time and are reproducible.

The field of sediment toxicity testing is very new relative to aquatic testing (particularly in the area of QA/QC), and considerable work will be required before the most effective QA/QC approaches are identified. Meanwhile, it is suggested that the principles outlined for aqueous testing in this document be adapted where possible to sediment tests.

2.6 Use of Two Versus One Reference Toxicant

Some proponents of reference toxicant testing recommend that laboratories employ two reference toxicants that demonstrate diverse modes or sites of toxic action (frequently an organic plus an inorganic chemical). The rationale is that abnormal test organisms might not show a significantly different test response to one chemical, but that tests using a different chemical could detect the abnormal condition. This seems to be of greatest concern in effluent testing, where abnormal organisms can respond unusually to a particular effluent constituent while responding normally to a reference toxicant with a different mode of action.

The database in the literature is inadequate to assume that any given reference toxicant will be particularly effective in detecting abnormal organisms (Section 2 and Appendix A). The few published studies have demonstrated that even where tests differentiated between groups of fish, the relative change from the initial end point was less that 75 per cent

(Dorn and Rodgers, 1978; Adelman and Smith, 1976; Hansen *et al.*, 1979). Therefore, with respect to a warning chart, a change in organism health may not result in an outlying point.

In addition, there are substantial sources of variability in both pure chemical and effluent tests besides organism health. For example, Dorn *et al.*,(1987) reported that the coefficients of variation associated with preparing reference toxicant test solutions were 15 to 136 per cent in two laboratories. However, an outlier caused by this source variability would not be directly relevant to effluent tests due to differences in solution preparation techniques between the two test types.

It is inappropriate to place too much weight on a reference toxicant test to identify the specific factors that may influence an effluent test result; the reference toxicant test results provide one general indicator of performance in a given test system. Rigorous attention should also be directed to other QA/QC activities, such as blank controls during tests, test replication, round-robin testing, and monitoring the health/growth/reproduction of test organism stocks and cultures. Records of all QA/QC activities should be reviewed whenever an effluent or a reference toxicant test appears unusual (see Section 5.2).

Section 3

Chemical Aquisition and Handling

All recommended reference toxicants (Section 2) are readily available in high purity and are inexpensive (Table 3). The suppliers presented in Table 3 do not represent an exhausive list of sources. Prices and catalogue numbers (quoted for December 1989) are presented for comparison and identification only.

Chemicals are typically obtained by telephoning a local supplier and requesting a specific chemical and quantity. Delivery may take as little as a few days (if the chemical is in stock) or several weeks.

Recommended procedures for handling and storage vary from chemical to chemical; details are provided for each chemical in Appendix B. In general, protective clothing is always advisable (e.g., gloves, safety goggles), skin contact should be avoided, and inhalation of harmful vapours should be prevented. All chemicals should be stored in well labelled containers, in a cool, dry, ventilated area, away from reactive materials or flame.

Table 3 Chemical Suppliers

Chemical	Supplier*	Purity	Quantity (g)	Cost ($)**	Cat. No.**	Special Considerations***
4-Chlorophenol	BDH	(99.5%)	500	83.62	B27730-34	Strong odour. Ensure adequate ventilation.
	Canlab		100	22.71	2304-100	
	Fisher	Reagent	500	28.53	1052299	
Phenol	BDH	AnalaR (99.5%)	500	81.00	B10188-34	Severe eye and skin irritant. Stable if not exposed to light or air
	Canlab	ACS	500	21.59	0028-500	
	Fisher	ACS	500	26.55	A92-500	
Sodium Penta-chlorophenate	Aldrich	(93.0%)	1000	17.90		Highly toxic carcinogen.
Cadmium ($CdCl_2$)	BDH	(95.0%)	250	88.50	B27549-32	Avoid contact or inhalation. Probable carcinogen or teratogen.
	Canlab	ACS (99.0%)	500	75.31	3996-500	
	Fisher	ACS (99.0%)	500	53.10	C10-500	
Chromium (K_2CrO_4)	BDH	AnalaR (99.5%)	500	85.00	B10199-34	Harmful skin adsorption can occur. Avoid inhalation. Carcinogenic.
	Canlab	ACS	500	25.47	6870-500	
	Fisher	ACS (99.5-100.5%)	500	22.91	P220-500	
Chromium ($K_2Cr_2O_7$)	BDH	AnalaR (99.9%)	500	84.98	B10202-34	Harmful skin adsorption can occur. Avoid inhalation. Carcinogenic.
	Canlab	ACS (99.95-100.5%)	500	45.84	6772-500	
	Fisher	ACS	500	23.76	P188-500	
Copper ($CuSO_4$)	BDH	AnalaR (99.5%)	250	50.08	B10373-32	Hygroscopic.
	Canlab		500	51.17	4848-500	
	Fisher	(97.0%)	500	31.64	C495-500	
Potassium Chloride	BDH	AnalaR (99.5%)	500	24.91	B10198-34	Avoid inhalation of dust.
	Canlab	ACS	500	9.78	6858-500	
	Fisher	ACS	500	10.80	P217-500	
Sodium Chloride	BDH	AnalaR (99.9%)	500	18.38	B10241-34	
	Canlab	ACS	500	7.60	7581-500a	
	Fisher	ACS	500	8.27	S271-500	
Zinc ($ZnSO_4$ heptahydrate)	BDH	AnalaR (99.5%)	500	52.91	B10299-34	
	Canlab	(99.0-103.0%)	500	14.88	8880-500a	
	Fisher	(99.0-103.0%)	500	13.59	Z68-500	
Silver Nitrate	BDH	ACS		392.00	ACS744-34	Caustic. Corrosive to skin, eyes, and mucous membranes. Incompatible with many chemicals.
	Canlab	ACS (99.9%)		108.76	7992-4	
	Fisher	ACS		123.43	S181-500	

* Canlab, A Division of Travenol Canada Inc.; Fisher Scientific; BDH Inc.; Aldrich Ltd.

** As of December 1989.

*** All chemicals must be handled with at least the minimum precautions including gloves, safety goggles, and dust mask (see Appendix B). Additional precautions are indicated.

ACS - Indicates that the chemical meets the requirements of the American Chemical Society Committee on Analytical Reagents.

AnalaR - Analytical reagent grade.

Reagent - Reagent grade.

Section 4

Conducting Tests

Reference toxicant tests should be conducted according to the specific test protocols used by the toxicity laboratory. This document is intended as a companion manual for Environment Canada's recommended Biological Test Methods, although the procedures described below can be applied to any recognized protocol. The test methods are not reiterated here; only the elements of testing, analysis, and interpretation that are specific to reference toxicant testing are provided.

Methods for accurate test solution preparation are outlined in Appendix E.

All procedures during a test are subject to a specified test protocol. Warning charts that are prepared for a specific test type should include data derived from a single test protocol.

4.1 Testing Frequency

Ideally, reference toxicant tests would be conducted continuously for each test type that is being performed by the laboratory, in order to minimize the time lag involved prior to detection of an abnormal condition. This frequency is impractical in most laboratories, however. The appropriate testing interval should be determined by experience gained in developing a base of reference toxicant data (e.g., after 15 to 20 tests). Monthly testing is recommended as a minimum. For organisms that are not cultured in the laboratory, and additional stipulation is that all stocks be tested upon arrival and just prior to exhaustion of the stock to determine whether:

a) the stock sensitivity to the reference compound is similar to that of previous stocks; and

b) the sensitivity of the stock to the reference toxicant changed significantly during holding in the laboratory.

Reference toxicant tests should be conducted more frequently when new organisms or protocols are introduced into the laboratory in order to establish warning limits early in the program. Weekly tests are probably not unreasonable for acute toxicity testing and bi-weekly for chronic toxicity testing in the initial few months. Once approximately five to ten tests have been completed with consistent results (e.g., a coefficient of variation in the end point of less than 30 per cent, as a guideline), the frequency can be decreased. All laboratories would be well advised not to report the findings of new tests until consistent reference toxicant test results can be demonstrated.

4.2 Chemical Confirmation of Test Solutions

Periodic confirmation of test solutions upon makeup is necessary to ensure that accurate toxicant concentrations can be achieved. Samples should be collected from a low, medium, and high concentration in each type of test at least two times per year (approximately once every six tests) to

confirm that actual concentrations are acceptable representations of nominal concentrations. Also, samples of exposure solutions should be collected every time and analyzed if data are out of control. The degree of difference that may be considered acceptable will depend on the analytical precision for that chemical at different concentrations. For example, if the highest concentration that can be analyzed by an instrument is three orders of magnitude less than the test solution concentration, the analyst will need to dilute the solution to bring the test concentration down to the working range of the instrument. Each dilution will compound any analytical error, resulting in poor analytical precision and poor resolution of test solution accuracy. Conversely, if a test solution is within the working range of concentrations for an analytical instrument, an accuracy of one or two per cent of the nominal concentration is achievable (up to five per cent is reasonable). It is advisable to consult with the analytical laboratory to determine the level of difference between nominal and measured concentrations that can be considered significant. The toxicity laboratory should select a reference toxicant for which it can be demonstrated that the accuracy of solution preparation is ten per cent or less.

To ensure that each technician in the laboratory is capable of preparing accurate solutions, stock solutions should be prepared by each person at least once annually and submitted for analysis. New personnel should be required to submit a toxicant solution for chemical confirmation early in their training period. Again, the acceptability of each result will depend in part on the uncertainty associated with the analytical method.

Unacceptable deviations in measured concentrations from expected concentrations will require a thorough investigation to identify the source of error. Calculation errors, dilution errors, poor accuracy in instrumentation and equipment are potential factors in inaccurate solution concentrations.

Section 5

Warning Charts

The discussions in Sections 5 and 6 assume a working knowledge of basic statistical functions and concepts (e.g., mean, standard deviation, confidence intervals, linear regression, significant difference). The necessary familiarity with statistical methods may be acquired by reading some basic texts on statistical concepts (e.g., Ostle and Mensing, 1975; Bhattacharyya and Johnson, 1977).

5.1 Establishing and Updating Warning Charts

Reference toxicant tests are used to demonstrate the ability of laboratory personnel to obtain consistent, precise results with a given test organism and protocol. This is accomplished using the same techniques that have been developed by chemical analytical laboratories over the past 20 years. One of these techniques is the mean chart.

The mean chart is prepared for reference toxicant tests by plotting the results of a successive series of tests on a chart where the x-axis represents the test date or test number and the y-axis indicates the end point concentration. In acute toxicity tests the end points are usually LC_{50}s* and EC_{50}s** which are continuous variables that are usually reported with an associated confidence interval. In chronic toxicity tests, a common method of analysis involves hypothesis testing, which produces endpoints that can only be one of the tested concentrations (i.e., the NOEC or LOEC) or the geometric mean between the two (i.e., chronic value). These endpoints are discrete variables and are not as appropriate for charting. An alternative end point in chronic tests is a continuous variable termed the IC_{50}*** (Norberg-King, 1988), which also gives confidence limits.

The mean and standard deviation of a set of reference toxicant test data can be used to define a range of "normal" or "acceptable" variability in the test. For example, the mean LC_{50} (arithmetic or transformed, see below) and standard deviation can be calculated for a series of acute lethality tests with rainbow trout within a single laboratory over a period of time. Given a sufficiently large sample size (e.g., 15 to 20 data points), the concentrations that equal two times the standard deviation above and below the mean ($\bar{x} \pm 2$ SD) represent the upper and lower 95 per cent confidence limits, respectively, for that data set. These lines ("warning limits") are then plotted on the mean chart (Figure 3).

At the 95% confidence level, 1 in 20 analyses (five per cent) would be expected

* LC_{50} is the median lethal concentration (i.e., the concentration of material in water that is estimated to be lethal to 50 percent of the test organisms after a fixed period of exposure).

** EC_{50} is the median effective concentration (i.e., the concentration estimated to cause a specified non-lethal or lethal effect on 50 percent of the test organisms after a fixed period of exposure).

*** IC_{50} is the median inhibition concentration (i.e., the concentration estimated to cause a specified inhibitory effect on 50 percent of the test organisms after a fixed period of exposure).

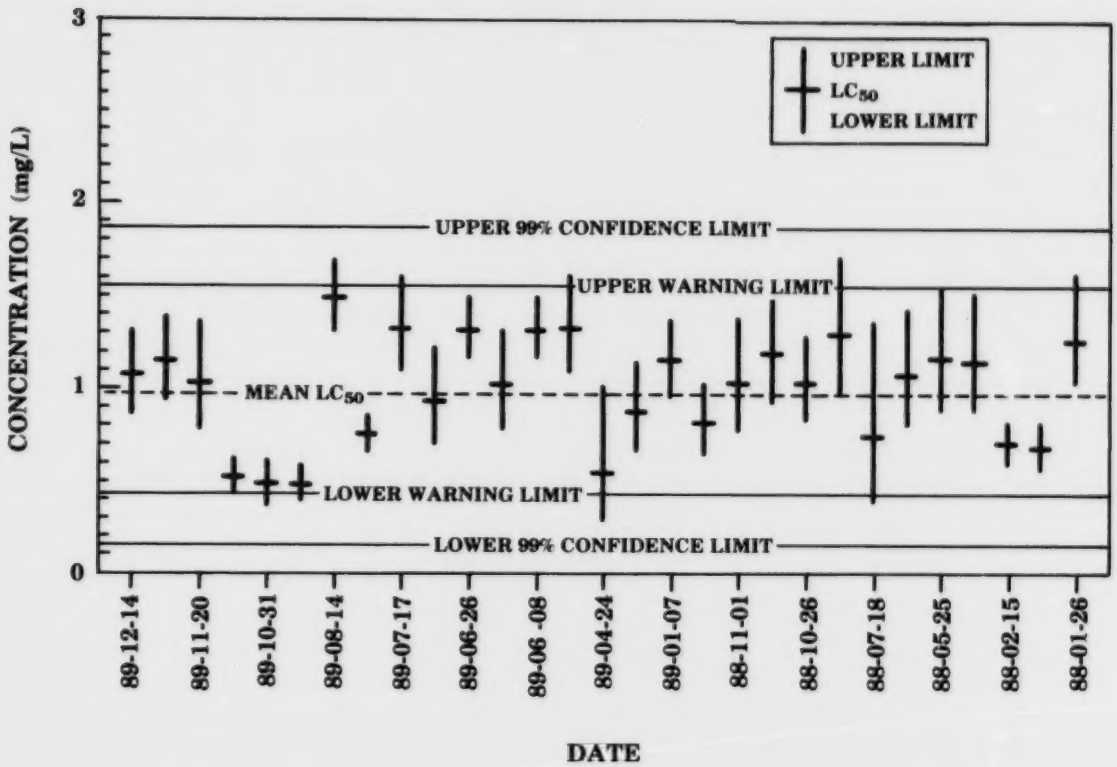

Figure 3 Mean Chart

to fall outside of the limits by chance alone. Interpretation of outlying data is discussed in Section 5.2.

The concentrations which equal the mean plus or minus three times the standard deviation ($\bar{x} \pm 3$ SD) represent the 99.7 per cent confidence limits (which are referred to hereafter as 99 per cent). At this confidence level, the probability of data falling outside of the limits by chance alone is only 0.3 per cent (one out of every 333 tests). While not necessary, inclusion of the 99 per cent limits on the warning chart is useful in interpreting the severity of outlying data. Severe outliers (outside 99 percent limit) should not be used in any subsequent recalculation of limits.

One of the assumptions underlying the statistics previously described is that a sufficient number of tests has been properly conducted to give a representative range of variability. To be certain that this is the case, 15 to 20 tests may be necessary (Dux, 1986). This may require considerable time (particularly for chronic tests), and the toxicity laboratory will probably want to estimate the test precision prior to that time. The U.S. EPA requires that a minimum of five tests be conducted before 95 per cent limits are established (Weber *et al.*, 1989). The laboratory should be aware that until a large number of tests have been completed, the limits are likely to change with the addition of each new data point to the data set. The limits will stabilize over time.

Another statistical assumption is that the data are normally distributed. The Kolmogorov-Smirnov test for normality (Sokal and Rohlf, 1981) should be performed prior to establishing a control chart. If the raw data are normally distributed, the arithmetic mean and standard deviation are used. Otherwise, a transformation must be performed to normalize the data. Experience has shown that log transformation will usually result in normality of non-normal LC_{50} data. Such data may be charted on the transformed or original scale. If an arithmetic scale is used, the control chart will show the logarithmic (geometric) mean of the data, and the associated 95 per cent and 99 per cent confidence limits will not be equidistant about the mean.

If a logarithmic transformation does not result in data normality, a suitable transformation must be found. The laboratory may want to use the maximum likelihood method of Box and Cox (1964) to choose an optimum transformation.

Separate control charts should be prepared for each reference toxicant-test, species-test protocol combination. Each new test result (LC_{50}, EC_{50}, or IC_{50}) should be compared against established warning limits and, if it falls within the limits, included in the data set. Interpretation of unusual or outlying data is discussed in Section 5.2.

Another consideration in the establishment of control limits is that data that show a high degree of variability will result in a large standard deviation about the mean, causing control limits to be wide. Therefore, although a laboratory may not be generating consistent results, they may be able to demonstrate that the data are within the warning limits. No accepted standards regarding the width of the 95 per cent confidence limits have been found among regulatory authorities that have implemented reference toxicant testing requirements (e.g., the U.S. EPA). Based on discussions with scientists in Canada and the U.S.A. (Appendix C), an objective coefficient of variation (%CV = 100 SD/$\bar{x}$) of 20 percent for each test is suggested. It is recognized, however, that such factors as the degree of standardization of each test protocol will also affect test reproducibility. A higher CV (e.g., 30 per cent) may be more realistic for some tests. It will not be possible to set specific limits on the width of control limits until sufficient data have been collected from laboratories across Canada demonstrating the degree of reproducibility that *can* be achieved.

The data should be stored electronically using spreadsheet software such as Lotus 1-2-3 or Multiplan to facilitate re-calculation of the mean and standard deviation for each data set. The charts can be plotted manually, but can be more conveniently plotted and updated using commercially available software packages such as Microsoft Chart, Lotus Freelance, or Harvard Graphics.

5.2 *Data Interpretation*

5.2.1 Warning Limits

As discussed previously, at the 95 per cent confidence level, five per cent of the test results would be expected to fall outside of the warning limits due to chance. An outlier should prompt a review of the test system. A mistake in stock solution preparation, a dilution calculation error, or stressed or undernourished test organisms are only some of the possible factors. It is particularly important to examine other QA/QC measures in the laboratory. Control survival during the tests, reproductive success of cultured organisms, time to first

brood and size of first brood in invertebrate cultures (and in tests when appropriate), dissolved oxygen levels, test temperature, etc., will provide important clues as to whether the outlier occurred by chance or, more likely, was due to a change or problem in the test system.

If an outlier (of warning limits) can be attributed to a specific problem in the test system (e.g., dilution error, miscalculation of data, poor organism health) the data point should not be included in re-calculation of the limits. If the outlier appears to represent normal variability, it should be included in the data set (see Section 5.2.2).

Data from other tests of that type conducted during the period of time corresponding to that of the test may need to be flagged as suspect if the reason for the outlier is not identified, or if it is traced to a factor common to the other tests. For example, if an outlying reference toxicant result for *Daphnia magna* was attributable to poor culture conditions at that time (e.g., crowding), then other *Daphnia magna* tests may be suspect. Alternatively, if the outlier was traced to a mistake in the preparation of the stock solution, the results of concurrent effluent tests may be quite acceptable, since test solutions for the two tests (effluent vs. chemical) are prepared by different procedures. In either case, the test data in question (e.g., effluent test results) should be reported with a note detailing the reference toxicant test results interpretation and other relevant QA/QC data. The reference toxicant datum that is to be reported with each set of routine test data should be that generated by the most recent reference toxicant test. The period of time for which each reference toxicant datum applies is therefore dependent on the chosen testing frequency (Section 4.1). Over time, the frequency of data falling outside the 95 per cent limits should be close to five per cent. If the frequency exceeds five per cent, miscalculation of the limits or a deterioration in precision is indicated. A frequency of less than five per cent may also indicate miscalculation of the warning limits or may demonstrate improved test precision. In the latter case, the laboratory may wish to re-establish the warning limits based on more recent data in order to more closely monitor and maintain the enhanced precision.

5.2.2 Confidence Limits

An outlier from the 99 per cent confidence limits is unlikely to occur by chance alone. The test system should be reviewed as outlined in Section 5.2.1. Even if a specific cause cannot be found to account for the outlier, it should not be attributed to chance. Concurrent data for that test system should always be flagged as suspect. The outlier should not be used in re-calculation of 95 per cent and 99 per cent confidence limits.

5.2.3 Data Trends

It is not only important to monitor whether or not each data point falls inside or outside established warning limits, but also to monitor trends or patterns that develop in the data. Out-of-control data may be prevented by early detection of a trend. Probability theory dictates that the probability of any single data point falling above or below the mean line is 50 per cent or 1/2 (assuming random sources of variation). The probability of two consecutive points being on the same side of the line is 25 per cent or 1/4.

The probability of "n" points being on the same side of the line is therefore $1/(2^n)$. If $n = 5$, the probability is only about three percent that this occurred through chance alone. Therefore, if five or more consecutive points are on the same side of the mean line,

some action should be taken to detect a source of bias (Dux, 1986).

5.2.4 Training New Technicians

Reference toxicant tests can be used to judge the progress of new personnel. New technicians should be required to conduct a series of reference toxicant tests until they are able to demonstrate the ability to consistently generate results within established warning limits.

Section 6

Duplicate Testing

Although duplicate testing is not a requirement of reference toxicant testing, it is a component of laboratory QA/QC that can be built into a reference toxicant testing program. Since the laboratory will usually be conducting reference toxicant tests once every two weeks to a month (Section 4.1), duplicate reference toxicant testing is probably only practical at a frequency of three to four times per year. Duplicate tests, however, should be performed in approximately one out of every ten routine tests in the laboratory. It is appropriate to perform duplicate tests on other types of samples analyzed in the laboratory and to document the results separately from the duplicate reference toxicant test data. For example, since the majority of tests conducted by many toxicity laboratories use effluent samples, it is appropriate to duplicate effluent testing on those samples.

Two alternative ways by which duplicate effluent test results can be analyzed, and one method for reference toxicants, are discussed below. The laboratory may choose whichever method is most appropriate. A limitation to both effluent methods is that the data points (unless individually identified as a letter, special symbol or date, for example) do not indicate temporal changes. It is advisable to review the data after every update to identify temporal trends that could indicate that the data are moving toward an out-of-control condition.

6.1 High vs. Low Duplicate Plots

This method is used for effluent test control, when considerable variation in sample toxicity can occur. Duplicate test results (e.g., LC_{50}, EC_{50} or IC_{50}) are plotted with the lower value on the x-axis and the higher value on the y-axis (Figure 4). If the slope of the regression line fit to this plot is 1, then the y-intercept represents within-laboratory test variation, i.e.:

$$S = \frac{intercept}{\sqrt{2}}$$

If the slope is significantly different from 1, then there is a systematic change in reproducibility with changing toxicity. In either case, a control limit can be calculated for the data (LC_{50} {from 1:1 line} + {R x 3.267}), where R equals the expected range (high minus low LC_{50} at a given LC_{50} value), and 3.267 is adopted from standard control charting practices (King, 1984; ASTM, 1986).

Tests outside the control limit would be suspect. As with mean charts, the statistics are based on the assumption that the data set contains a sufficient number of representative samples (e.g., 15 to 20), and that residuals from the regression line follow a normal distribution.

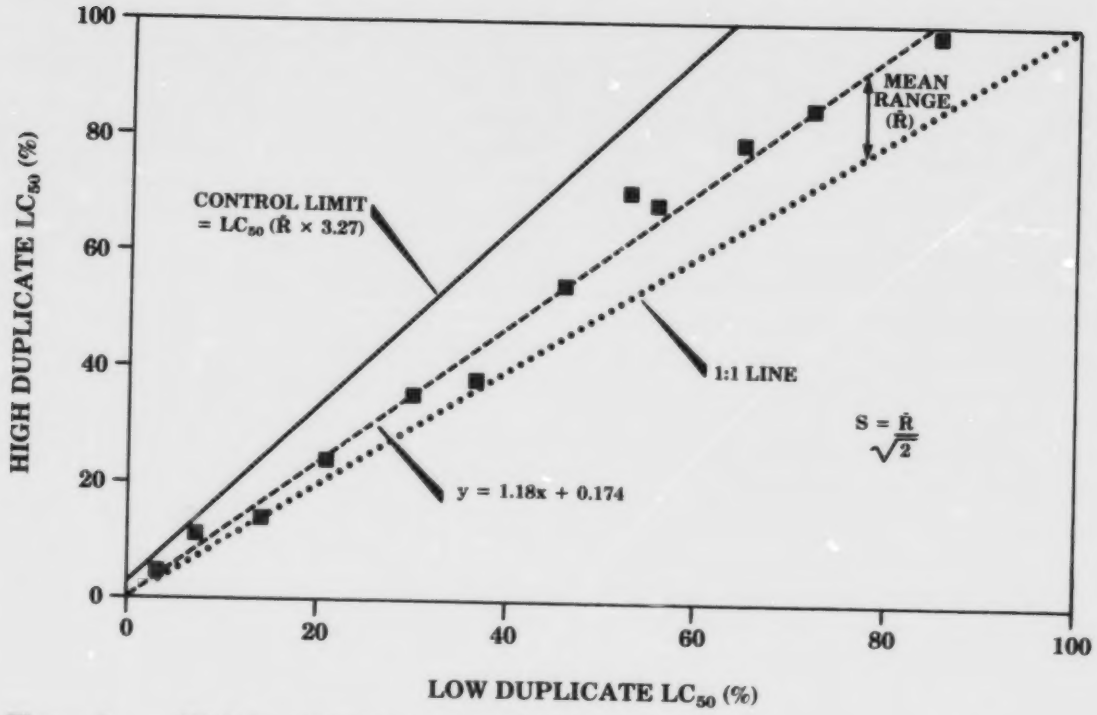

Figure 4 High-Low Duplicate Control Chart

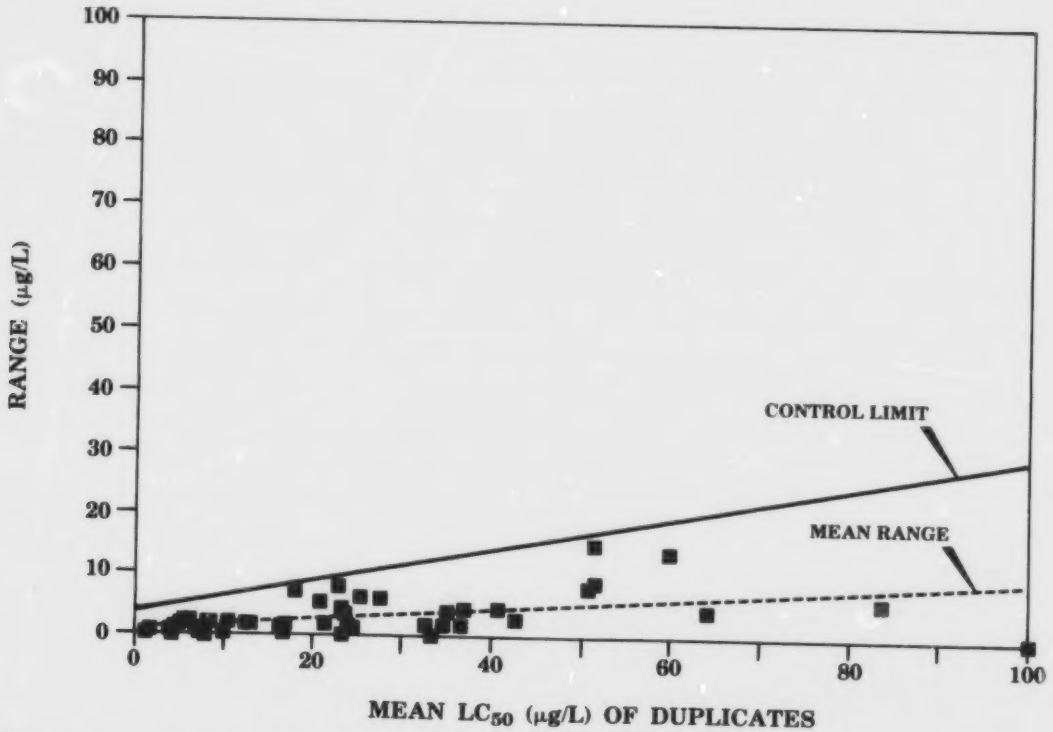

Figure 5 Range-Type Control Chart

6.2 Range-Type Control Charts for Effluents

To set up a range-type control chart, the range for each pair of endpoints (e.g., LC_{50}) should be plotted against the mean end-point value. The range of difference between duplicates is often somewhat dependent on end-point concentration when samples vary in toxicity. Control limits can be adjusted for this effect. A regression line gives the expected range at a given end-point concentration. A control limit is calculated as 3.267 x expected range (King, 1984; ASTM. 1986) and is presented as in Figure 5.

6.3 Range-Type Control Charts for Reference Toxicants

When the end point is expected to be relatively constant, as in a reference toxicant test, there should be no relationship between duplicate range and end-point concentration, and ranges can be charted in temporal sequence, rather than against end-point concentration (cf. Figure 3). This approach facilitates early detection of developing trends in test precision. The mean range ($\bar{R}$) provides the central line of the control chart, and a control limit is calculated as $3.267 \times \bar{R}$ (King, 1984; ASTM, 1986).

Section 7

Record-keeping and Data Reporting

The raw data sheets (bench sheets) for each test type should be filed together and kept in a central, easily accessible location. It is extremely important that all relevant records be included on the bench sheets (paper, electronic or both) such as test date, stock solution preparation date, unusual conditions, test technician, test data, etc. (see Environment Canada, 1990a and 1990b, and Hart, 1989, for others). Thorough documentation can reduce the time and expense associated with tracing the source of out-of-control data.

There should be a QA officer in charge of monitoring and updating laboratory QA/QC procedures. The QA officer should also be responsible for scheduling the reference toxicant tests. The schedule should comply with the requirements discussed in Section 4.2 and may also incorporate duplicate reference toxicant testing requirements as outlined in Section 6.

Once a reference toxicant test is complete and has been analyzed, the data should be checked by the laboratory supervisor. Bench sheets and results should then be passed on to the QA officer for comparison with existing control limits. Warning charts should then be updated to include the new data, provided that the data are within control limits. Outlier data should trigger an immediate investigation. As discussed in Section 5.2, the investigation may indicate that other data obtained using the same protocol are suspect. In this event, it would be desirable for the toxicity laboratory to repeat the suspect tests after corrective action is taken. This is usually impossible, however, due to limited sample volumes and/or sample aging. Alternatively, the toxicity laboratory should report the results of the reference toxicant test, with all suspect data, including an interpretation of the results as to data quality.

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Appendix A

Reference Toxicant Selection

The first step of a two-step process involved identifying the fundamental characteristics that would be important in achieving the reference toxicant testing objective (Section A.1, below). The second phase involved examining the suitability of the toxicants that passed the first (screening) phase as reference toxicants in specific types of tests (e.g., fish, invertebrates, algae, acute vs. chronic, marine vs. freshwater) (Section A.2).

It was recognized that much of the information that was necessary in evaluating the suitability of chemicals as reference toxicants may not be available in the public domain. A number of scientists from Canada and the U.S.A. were contacted and asked to provide any relevant comments and/or data. A list of persons that were able to assist in this evaluation is presented in Appendix C. Reference to the persons in this document has been noted either as "personal communication" for verbal, or unpublished data if data were provided for the authors' review.

A.1 General Selection Criteria

The criteria considered to be important in identifying suitable reference toxicants have varied between authors (Alderdice, 1963; Adelman and Smith, 1976; LaRoche *et al.*, 1970; Fogels and Sprague, 1977), depending largely on each author's perception of the underlying purpose(s) of the tests. Eight primary selection criteria were identified as fundamental characteristics, in order for reference toxicant tests to be used as a measure of test reproducibility (precision) within a laboratory they must :

a) have been successfully used to detect abnormal organisms;

b) have an established toxicity database (i.e., to provide confirmation of observed results);

c) be readily available in pure form;

d) be readily water soluble (i.e., to avoid use of a carrier);

e) be stable in solution (i.e., so that toxicity results will not be affected by rate of toxicant dissipation in different tests; also to facilitate chemical confirmation of exposure concentrations);

f) have a stable shelf life (i.e., toxicant strength does not change);

g) variations in water quality that may be expected within a given laboratory have only limited effects on toxicity test results over time; and

h) be easily analyzed.

The chemicals evaluated have had some history of use as reference toxicants; two others are currently being evaluated as reference toxicants for use by the U.S. EPA (potassium chloride) and the Ontario Ministry of Environment (4-chlorophenol). Other chemicals have been suggested (e.g., DDT, endosulfan, picloram) or could be suggested as potential reference toxicants, but the evaluation was limited to those

chemicals for which information relevant to reference toxicant testing could be found.

Only zinc received the ideal score of eight (Table A.1; supporting references on Tables A.2 to A.12). The low scores for DSS and $AgNO_3$ caused them to be eliminated from further evaluation.

A.2 Reproducibility of Toxicity Test Results using Various Reference Toxicants

Only chemicals that met most of the necessary selection criteria (A.1) were reviewed.

A coefficient of variation (CV) of 30 per cent (where % CV = (standard deviation/ mean) x 100) was used to judge acceptable reproducibility of results in the absence of any historically accepted standards. The 30 per cent figure was selected based on discussions with experienced toxicologists that were contacted (Appendix C) as well as the authors' laboratory experience in reference toxicant testing (see also Section 5.1). A reference toxicant that typically gave test results with a CV of 30 per cent or less in repeated intralaboratory tests was judged acceptable for that particular test type. Interlaboratory test results of 30 per cent or less were also judged acceptable since interlaboratory results would likely demonstrate lower rather than higher variability.

No judgement was made (indicated by "?", Table A.13) if no data or published references were available.

A.2.1 Organic Chemicals

A.2.1.1 4-chlorophenol

Fish - Acute Tests

- K. Doe, unpublished data - Two tests (different dates) using each of rainbow trout and threespine sticklebacks gave consistent results; LC_{50}s and confidence limits for trout were identical and those for sticklebacks gave CV of 20 per cent.

Fish - Chronic Tests

- No data found.

Invertebrates - Acute Tests

- D. Poirier, unpublished data - Thirteen tests over six months using *Daphnia magna* gave CV of 25 per cent; no drop in dissolved oxygen during tests.

- K. Doe, unpublished data - Six tests with *Daphnia magna* give CV of 21.7 per cent; in a seventh test, an LC_{50} could not be calculated due to lack of dose-response relationship; some degree of inverse dose-response observed in most tests.

Invertebrates - Chronic Tests

- No data found.

Algae Tests

- No data found.

Marine Tests

- No particular problems found to be associated specifically with tests conducted in marine rather than fresh water.

Microtox ™

- No data found.

A.2.1.2 Phenol

Fish - Acute Tests

- Klaverkamp *et al.* (1975) - Phenol was rapidly toxic to rainbow trout; highly soluble, readily available; non-specific mode of toxic action, in continuous-flow tests, measured concentrations were 75 to 99 per cent of nominal.

- Walker (1988) - Variability between phenol acute toxicity tests conducted in different laboratories and under widely varied conditions (e.g., continuous-flow, temperature, pH, hardness) showed surprisingly low coefficients of variation (CV); CV for fathead minnow LC_{50} = 38 per cent (N = 10 tests in 6 studies); CV for rainbow trout = 39 per cent (N = 3 tests in 3 studies).

- Fogels and Sprague (1977) - Threshold of acute lethality for rainbow trout, zebrafish and flagfish achieved quickly; phenol satisfied all criteria considered by authors for reference toxicant selection (e.g., easily analyzed, soluble, lethal in low mg/L range, readily available, pKa at least one unit removed from pH of dilution water, known mode of action, definite lethal threshold, minimal water quality effects).

- Alexander and Clarke (1978) - Rainbow trout responses to phenol were the most sensitive of five reference toxicants tested to stresses induced by starvation (LC_{50} and median survival time (MST), temperature (MST), or chlorine (LC_{50} and MST); differences between some genetic strains observed (MST and LC_{50}); no differences resulted during crowding stress or elevated holding mortality, or from different diets.

- U.S. EPA (1980f) - Three acute tests conducted using bluegill produced LC_{50}s with CV of 30 per cent ($\bar{X}$ = 15 mg/L).

- K. Doe, unpublished data - Nineteen acute rainbow trout tests gave CV of LC_{50}s of 13.6 per cent; no loss of chemical occurred in solutions aged for 0.5 hour to 4 days prior to introduction of fish, indicating low bacterial activity in dilution water; after addition of fish, chemical levels dropped rapidly; six acute tests conducted using threespine stickleback gave LC_{50}s with CV of 31 per cent.

- J. Somers, unpublished data - Sixty-eight rainbow trout tests gave CV of LC_{50}s of 17.3 per cent; differences in fish stocks observed using phenol tests; a nutritional problem also emerged over time through routine tests; most lethality occurred within 24 hours during tests.

- Dalela *et al.* (1980) - Acute tests using three freshwater teleosts (*Notopterus notopterus, Colisa fasciatus* and *Saccobranchus fossilis*) resulted in significant differences ($p < 0.05$ to $p < 0.001$) in 96-hour LC_{50}s between tests conducted at pHs of 6.0, 7.3 or 8.8; however, LC_{50}s increased by factors of only 1.1 to 2.2 from pH change 6.0 to 7.3 or 7.3 to 8.8.

Fish - Chronic Tests

- No data found. Phenol may be suitable for chronic tests as in acute tests, since solutions are replaced every day.

Invertebrates - Acute Tests

- U.S. EPA (1980f) - Acute tests conducted using *Daphnia magna* (N = 5) and *D. pulex* (N = 5) gave EC_{50}s with CVs of 4.9 and 6.5 per cent, respectively ($\bar{x}$ = 92 and 85 µg/L, respectively).

- Walker (1988) - Variability between *Daphnia magna* tests conducted in different laboratories and under different conditions (e.g., temperature, measured

vs. unmeasured chemical) was great; CV of LC_{50}s was 118 per cent.

- K. Doe, unpublished data - Twelve *Daphnia magna* tests conducted over a three-month period gave low CV of LC_{50} of 28 per cent.

- D. Poirier, personal communication - Dissolved oxygen drops too low in *D. magna* tests using phenol resulting in erratic mortality.

Invertebrates - Chronic Tests

- No data found. Phenol may be suitable for chronic tests since solutions are replaced daily.

Algae Tests

- D. St-Laurent, unpublished data - CV of 12.7 per cent achieved in three tests using microplate technique (mean EC_{50} = 68.8 mg/L); all tests conducted by same analyst but on different days with different solutions and algal lots.

Marine Tests

- No problems found (or anticipated) to be associated specifically with marine versus freshwater tests.

Microtox ™

- Curtis *et al.* (1982) - One set of duplicate tests gave CV for five-minute LC_{50}s of 1.2 per cent.

- Dutka *et al.* (1986, as cited in E.V.S., 1989) - Microtox assays gave coefficient of variation of 2.4 per cent (no N cited).

- Qureshi *et al.* (1982) - One set of duplicate tests gave CV for five-minute EC_{50}s of 24 per cent.

A.2.1.3 Sodium Pentachlorophenate

Fish - Acute Tests

- Davis and Hoos (1975) - Interlaboratory study of seven labs in B.C.; static tests using juvenile rainbow trout, coho salmon and sockeye salmon; standard stock solutions; LC_{50}s for the three species ranged from 37 to 130 µg/L and the authors concluded that the differences could largely be attributed to pH of laboratory waters.

- Fogels and Sprague (1977) - Toxicity threshold achieved within the 96 hours, continuous flow tests using rainbow trout, zebrafish and flagfish.

- Adelman and Smith (1976) - Eight acute tests were conducted in duplicate using both fathead minnows and goldfish; CVs of 96-hour LC_{50}s were 12 and 17 per cent (for each species), respectively; most mortality occurred within 24 hours.

- Dalela *et al.* (1980) - Acute tests using *Notopterus notopterus, Colisa fasciatus,* and *Saccobranchus fossilis* showed significantly different LC_{50}s ($p < 0.01$ and $p < 0.001$) between tests conducted at pH 7.3 and pH 8.8 for each species; resulting increase in LC_{50}s ranged from 1.9 to 4.5 times for the three species; pH changes from 6.0 to 7.3 resulted in LC_{50} increases of 1.3 to 1.7 times for the three species with the change being significant for two of the three.

- Adelman *et al.* (1976) - Rate of fathead minnow mortality was similar for 4-, 7- and 11-week-old fish but different for 14-week-old fish; the threshold LC_{50} was similar for all groups; groups of small and large fathead minnows (all 11 weeks old) showed similar rates of mortality but different threshold LC_{50}s.

- BEAK, unpublished data - Seventy-one acute tests conducted over four years

using rainbow trout gave coefficient of variation of 22 per cent ($\bar{x}$ = 0.15 mg/L).

- Hansen *et al.* (1979) - PCP and NaCl tests were conducted over one year using six batches (stocks) each of rainbow trout, golden shiners, fathead minnows, and threespine sticklebacks, all tested in freshwater in duplicate. Pooled data from duplicates were combined to give one LC_{50} for each test; these LC_{50}s were then used by this author to give CVs for each batch (approximately four tests each batch) and an overall CV for each species over time (excluding tests which were invalidated by more that 20 per cent control mortality or those for which an estimate of LC_{50} could not be made). Rainbow trout gave within-batch CVs of 3 to 14 per cent but overall CV (of all tests, N = 19) was 29 per cent; most of the between-batch variability could be attributed to one batch that contained smaller fish and showed lower LC_{50}s; weekly tests with the same batch showed decreasing LC_{50} with decreasing condition of fish over four weeks. Golden shiners showed within-batch CVs of 1. to 19 per cent but overall CV was 24 per cent (N = 19); however, three of six batches showed 10 to 20 per cent holding mortality; therefore, variability between batches might be expected (even though no trend was established such as lower LC_{50}s in batches with higher holding mortalities). Sticklebacks also had high holding mortalities (28 to 33 per cent) in three of six batches with CVs of 3.2 to 25 per cent within-batch and 45 per cent overall (again, no correlation with holding mortality and LC_{50}). Simultaneous NaCl tests, however, were consistent within batches and between batches (i.e., except for sticklebacks, which showed high variability in both NaCl and PCP tests, CVs within and between batches were all less than or equal to 10 per cent except one [14 per cent]). NaPCP was possibly a more sensitive indicator of fish health.

Fish - Chronic Tests

- Pickering (1988) - NaPCP was one of the chemicals used to show a high degree of reproducibility between fathead minnow larval survival and growth tests and embryo-larval survival and teratogenicity tests (six tests); the ratio of the highest no-observed-effect concentration (NOEC) of all larval tests to the lowest NOEC was 2 (equal to the reciprocal of the dilution factor, e.g., they only differed by one test concentration); the CV of the chronic values was 39 per cent. The high/low NOEC ratio for the embryo-larval test was also 2; the CV of the chronic values was 34 per cent (note from this author that the variability associated with a non-continuous end point such as a chronic value may be different (likely higher) than a continuous end point); the CV associated with LC_{50} values from the embryo-larval tests was only nine per cent.

- DeGraeve *et al.* (1989) - Seven-day fathead minnow larval survival and growth tests were used to judge test performance in an interlab study; duplicate tests conducted on two days in 10 labs gave overall CV of the LC_{50}s of 44 per cent (spatial and temporal); mean CVs within the labs on the two days were 16 and 11 per cent; the combined data for both days showed total intralab variability of 43 per cent (spatial and temporal); LC_{50} differences were largely attributed to pH (significant positive correlation); pH changes during test (e.g., often more than 0.2 units) can alter results and reduce reproducibility.

- BEAK, unpubl. data - Ten (10) fathead minnow survival and growth tests conducted over eight months; CV of LC_{50}s was 15 per cent was 21 per cent

($\bar{x}$ = 0.36 mg/L); CV of IC_{50}s was 21 per cent ($\bar{x}$ = 0.35 mg/L).

Invertebrates - Acute Tests

- Canton and Adema (1978) - Five sets of duplicate tests involving three daphnid species and two laboratories showed very low intralab spatial variability (ratio of duplicate LC_{50}s ranged from 1.0 to 1.08); the ratio of LC_{50} values from one set of intralab temporal duplicates conducted at the same time gave an LC_{50} ratio of 1.67.

- Lewis and Weber (1985) - Intralaboratory precision estimates of *D. magna* and *D. pulex* tests (as the CV of 10 48-h LC_{50} tests for each organism) were 10 and 36 per cent, respectively.

- BEAK, unpublished data - Twenty-nine acute tests with *D. magna* conducted over two years gave CV of 29 per cent ($\bar{x}$ = 1.0 mg/L).

Invertebrates - Chronic Tests

- Weber *et al.* (1989) - Nine *Ceriodaphnia dubia* survival and reproduction tests produced chronic values ranging from 0.35 to 0.42 mg/L.

- BEAK, unpublished data - Seven *Ceriodaphnia dubia* survival and reproduction tests conducted over 10 months produced LC_{50}s with a CV of 30 per cent ($\bar{x}$ = 0.28 mg/L); the CV associated with the IC_{50}s was 14 per cent ($\bar{x}$ = 0.26 mg/L).

Algae Tests

- No data.

Marine Tests

- No particular problems found (or anticipated) to be associated specifically with marine versus freshwater tests.

Microtox ™

- Green and Bulich (1981, as cited in Bulich 1986) - Thirty determinations using Microtox gave CV for five-minute EC_{50}s of 11 per cent and for 15 minutes EC_{50}s of 12 per cent.

A.2.2 Inorganic Chemicals

A.2.2.1 Cadmium ($CdCl_2$)

Fish - Acute Tests

- Sprague (1987) - Toxicity of cadmium to salmonids, fathead minnows and sticklebacks shows results from two mechanisms: one dose-related and rapid-acting, and the second, a delayed violent overreaction to external stimuli; effects related to the second mechanism may be induced or enhanced by normal handling/monitoring procedures of test (see text, Section 2.2).

- U.S. EPA (1980a) - Five acute tests using fathead minnows conducted by a single lab gave CV of LC_{50}s of 59 per cent ($\bar{x}$ = 7180 µg Cd/L, from $CdSO_4$); another study showed a CV of 38 per cent in five tests which used mosquitofish ($\bar{x}$= 1740 µg Cd/L, from $CdCl_2$).

- K. Doe, unpublished data - Low test reproducibility; CV of four rainbow trout tests was 33 per cent; CV of six stickleback tests was 50 per cent.

- B. Peltier, personal communication - Not recommended for fish because they become narcotized and death is difficult to ascertain.

- Q. Pickering, personal communication - High variability and slow lethality.

Fish - Chronic Tests

- Weber *et al.* (1989) - Five fathead minnow survival and teratogenicity tests gave CV of LC_{50}s of 62 per cent.

- Sprague (1987) - As above.

Invertebrates - Acute Tests

- Lewis and Weber (1985) - Precision (CV) of *D. magna* and *D. pulex* acute lethality tests was 72.4 per cent (N = 8) and 20.9 per cent (N = 9), respectively.

- U.S. EPA (1980a) - Six acute tests conducted with the cladoceran, *Simocephalus serrulatus*, gave a CV of 81 per cent ($\bar{x}$ LC_{50} = 14 µg Cd/L from $CdCl_2$).

- Thomas *et al.* (1986) - Five tests using *Daphnia* sp. gave acute EC_{50}s with a CV of 20 per cent ($\bar{x}$ = 41 (µg/L).

Invertebrates - Chronic Tests

- No data found.

Algae Tests

- Weber *et al.* (1989) - In 11, 96-hour tests using *Selenastrum capricornutum*, EC_{50}s showed CV of 83 per cent.

- Thomas *et al.* (1986) - Five algae tests (no species specified) gave EC_{50}s with CV of 15 per cent.

Marine Tests

- No particular problems found to be associated specifically with marine rather than freshwater tests.

Microtox ™

- Thomas *et al.* (1986) - Five Microtox tests gave 5-, 15-, and 30-minute EC_{50}s with CVs of 40, 35, and 25 per cent, respectively ($\bar{x}$ = 106, 25, and 14 mg/L, respectively).

A.2.2.2 Chromium (as Potassium Dichromate, $K_2Cr_2O_7$, or Potassium Chromate, K_2CrO_4).

A discussion of the chemical characteristics of dichromate and chromate in aqueous solution was presented in Section 2. Both chemicals form part of the same aqueous equilibrium that involves formation of the most toxic form of hexavalent chromium, hydrochromate ($HCrO_4$-). Despite the slight differences between aqueous characteristics of the two chemicals (i.e., the effect of pH on formation of $HCrO_4$-), the information presented below for dichromate has been applied also to chromate since little relevant information was found for chromate specifically. Jop *et al.* (1986, 1987) demonstrated that the toxicity of the two chemicals (judged by acute LC_{50}s to marine and fresh water species) was very similar.

The following studies used dichromate unless otherwise indicated.

Fish - Acute Tests

- Jop *et al.* (1986) - Acute tests using *Cyprinodon variegatus, Pimephales promelas, Gasterosteus aculeatus* and *Lepomis macrochirus* gave CVs of 25 per cent (N = 5), 4 per cent (N = 2), 15 per cent (N = 2) and 7 per cent (N = 3), respectively; time-mortality curves showed that mortality was still occurring at 96 hours in many tests.

- Adelman and Smith (1976), Adelman *et al.* (1976) - Eight acute tests were conducted in duplicate with both fathead minnows and goldfish; CV for fathead minnow tests was 12 per cent while CV for goldfish tests was 38 per cent; fish mortality was initially (in 48 hours) rapid, slowed from 72 to 120 hours then

increased again after 120 hours; authors suggested that this indicated two modes of toxic action; the LC_{50} of an unhealthy fish stock (high holding mortality) was within the range of healthy fish; stock had been chemically treated and mortality had ceased prior to testing.

- K. Doe, unpublished data - Nine acute rainbow trout tests produced CV of 9.8 per cent; seven acute stickleback tests gave CV of 6.3 per cent; high concentrations resulted in dramatic shifts in pH; disposal of high concentrations was a concern.

Fish - Chronic Tests

- DeGraeve *et al.* (1989) - Variability of four fathead minnow survival and growth tests (duplicate tests on two days) investigated with 10 labs; interlaboratory variability averaged 24 per cent (as CV over all tests); mean CVs within labs on the two days were 10 and 6.9 per cent; combined data for both days showed total intralaboratory variability of 26 per cent (spatial and temporal variability).

- Anderson *et al.* (1989) - An interlaboratory study with 10 labs using the fathead minnow survival and growth test gave a CV of IC_{50}s of 31 per cent.

Invertebrates - Acute Tests

- Dorn *et al.* (1987) - Value of hexavalent chromium as a reference toxicant was investigated by comparing the precision of two laboratories in preparing test solutions and an inter- and intralaboratory comparison of results between two laboratories; CVs associated with preparing test solutions were 15 to 136 per cent (both tests at both labs); the 48-hour LC_{50}s for *Mysidopsis bahia* were not significantly different ($p < 0.05$) either within or between laboratories during the three-week study; acute toxicities of *D. pulex* differed significantly between laboratories by almost one order of magnitude (attributed to different food); CVs of *Daphnia pulex* tests in the two labs were 40 per cent (N = 6) and 93 per cent (N = 3), respectively; CVs in *M. bahia* tests were 24 per cent (N = 5) and 3.9 per cent (N = 3); one lab also tested potassium chromate using *Cyprinodon variegatus* (CV = 25 per cent, N = 4) and *Lepomis macrochirus* (CV = 16 per cent, N = 3).

- Jop *et al.* (1986) - Tests with *D. pulex* gave mean 48-hour EC_{50}s that showed a CV of 26 per cent; eight tests with *M. bahia* also gave consistent results (CV of 22 per cent); no significant difference was observed between a *D. pulex* test with chromate vs. dichromate.

- Vanhaeke *et al.* (1980) - Nine tests were conducted on one strain of *Artemia nauplii;* CV of LC_{50}s was 12.4 per cent.

- Dorn and Rodgers (1989) - Thirty-five *Daphnia pulex* tests conducted between September 1983 and August 1986; EC_{50}s showed CV of 32 per cent; a significant correlation ($r = -0.41$, $a = 0.05$) was observed between control mortality and acute toxicity of Cr^{6+}; when control mortality was between 12 per cent and 24 per cent in five successive tests (i.e., unacceptable), LC_{50}s were all less than the mean LC_{50} for all tests; since incident occurred early in establishment of culture, the authors concluded that the data indicated unhealthy organisms; 18 *Mysidopsis bahia* tests between September 1984 and July 1986 showed CV of EC_{50}s of 41 per cent; significant correlation ($r = -0.47$, $a = 0.05$) between control mortality and Cr^{6+} toxicity; tests with higher than 10 per cent control mortality were sometimes associated with more toxic response to Cr^{6+}

Invertebrates - Chronic Tests

- EPRI (1989) - Intralaboratory CVs for chronic LC_{50} estimated by four laboratories ranged from 18 to 55% while CVs for LC_{50}s ranged from 17 to 72%. As one or two tests were conducted per laboratory on two separate occasions, the CVs incorporate both spacial and temporal variability.

Algae Tests

- D. St.-Laurent, unpublished data - CV of 9.2 per cent achieved in three tests using microplate technique (mean EC_{50} = 65.7 µg/L as Cr^{6+}); all tests conducted by same analyst, but on different days, with different solutions and algal lot.

Marine Tests

- No particular problems found to be associated specifically with marine versus freshwater tests.

Microtox ™

- No data found.

A.2.2.3 Copper (as Copper Sulphate, $CuSO_4$)

- U.S. EPA (1980d) - The cupric ion is highly reactive and forms moderate to strong complexes and precipitates with many inorganic constituents of natural waters (carbonate, phosphate, amino acids, humates, suspended solids).

Fish - Acute Tests

- Sprague (1985) - Twenty-five acute rainbow trout tests conducted in a single laboratory by single analyst over two years gave LC_{50}s with a CV of 31 per cent, $\bar{x}$ = 304 µg/L; highest and lowest LC_{50} differed by only factor of 1.39; however, results from tests conducted by two other analysts three years previously (in the same laboratory using the same fish stock and water supply) gave LC_{50}s that differed from each other by a factor of 5.5; no explanation for the difference was evident.

- Fogels and Sprague (1977) - Toxicity threshold achieved within 96 hours for rainbow trout and zebrafish, but not for flagfish in continuous-flow tests.

- Alexander and Clarke (1978) - Rainbow trout stressed by starvation or crowding did not show significantly different survival times from unstressed fish exposed to $CuSO_4$; no difference detected between different rainbow trout strains.

- See Table A.14.

Fish - Chronic Tests

- U.S. EPA (1980d) - Unlike acute toxicity, chronic toxicity of copper to fish is not related to water hardness.

- See Table A.15.

Invertebrates - Acute Tests

- Thomas *et al.* (1986) - CV of four tests using *Daphnia sp.* was 10 per cent.

- See Table A.16.

Invertebrates - Chronic/Sublethal Tests

- Anderson *et al.* (1989) - CV of IC_{50}s from four oyster larvae development tests by different laboratories was 37 per cent; CV of IC_{50}s from six echinoderm fertilization tests by different laboratories was 66 per cent.

- See Table A.17.

Algae Tests

- U.S. EPA (1989a) - NOECs of marine alga, *Champia parvula*, ranged from

0.5 to 1.0 µg/L in six reproduction tests; LOECs ranged from 1.0 to 2.5 µg/L; natural seawater, 30 ‰.

- Thomas *et al.* (1986) - Four algae tests produced EC_{50}s with CV of nine per cent.

- D. St.-Laurent, unpublished data - CV of 8.1 per cent achieved in three tests using microplate technique (mean EC_{50} = 60.5 µg/L as Cu^{2+}); tests conducted by same analyst on different days with different solutions and algal lots.

Marine Tests

- Spear and Pierce (1979) - In marine waters the degree of inorganic complexation tends to increase with increased salinity and the predominant forms of dissolved copper are typically Cu^{2+} and $CuCl_2$. $CuCO_3$ and $Cu(OH_2)$ may also be abundant in marine waters, existing as colloidal dispersions. Complexation alters (typically reduces) toxicity to aquatic organisms.

- K. Doe, Q. Pickering, pers. comm. Problems (e.g., precipitation) reported in some marine waters.

Microtox ™

- Qureshi *et al.* (1982) - One set of duplicate tests gave CV of 26 per cent.

- Thomas *et al.* (1986) - Twenty five-minute Microtox tests resulted in EC_{50}s with CV of 49 per cent; 24 15-minute Microtox tests resulted in CV of 43 per cent; 25 30-minute tests resulted in CV of 30 per cent.

A.2.2.4 Potassium Chloride (KCl)

- G. Callous and B. Peltier, pers. comm. - No specific information was found regarding the reproducibility of results achievable with KCl in different test types. Researchers in the U.S. EPA are currently evaluating the suitability of KCl as a reference toxicant. Preliminary tests have given promising results. KCl has an advantage over many other toxicants of demonstrating minimal, if any, water quality effects on toxicity. Unlike NaCl, it would be suitable in marine tests.

A.2.2.5 Sodium Chloride (NaCl)

Fish - Acute Tests

- Adelman and Smith (1976), Adelman *et al.* (1976) - Low variability reported in eight duplicate acute tests using fathead minnows and goldfish (CV of 6 per cent for each species); most mortality occurred within 48 hours; 96-hour LC_{50} of an unhealthy goldfish stock (high holding mortality) fell well outside the range established for healthy stock (although test was not conducted until after mortality had ceased following chemical treatment of water containing diseased fish).

- Hansen *et al.* (1979) - NaCl was used as a reference toxicant with rainbow trout, golden shiners, fathead minnows, and sticklebacks in freshwater over one year; six stocks of each species used during the year; data from duplicate tests was pooled to give single LC_{50} for each test; 18 rainbow trout tests produced LC_{50}s with CV of 10 per cent; one stock was substantially smaller than others but NaCl tests resulted in LC_{50}s similar to other stocks; NaPCP tests on the same fish showed lower LC_{50}s than other stocks; also, four weekly tests on the same stock with PCP showed decline in both LC_{50} and condition factor; sticklebacks showed high control mortality (28 to 33 per cent)

in four of six batches of fish; CV of 23 stickleback LC_{50}s was 29 per cent; golden shiners had 10 to 20 per cent mortality in three of six batches but CV of all acute tests was only 5.9 per cent; holding facilities for all species were crowded and preventative disease treatment (chemical) was necessary during holding.

- T. Kovacs, personal communication - Consistent results achievable in acute tests using rainbow trout and fathead minnows; one batch of diseased trout (fungus) showed LC_{50} within acceptable range; rapid mortality (mostly less than 48 hours).

Fish - Chronic Tests

- Taraldsen *et al.* (1989) - Suitable for chronic fathead minnow tests; consistent, reproducible (no data given); no pH or hardness effects on toxicity.

- T. Norberg-King, personal communication - Very reproducible results achievable with fathead minnows; NOEC usually only varies by a factor of 2 (e.g., one dilution in dilution series of 0.5).

Invertebrates - Acute Tests

- Taraldsen *et al.* (1989) - Suitable reference toxicant for *D. magna* and *D. pulex*; reproducible (no data given); no hardness- pH effects.

- T. Kovacs, personal communication - Consistent results achievable with *D. magna* and *Ceriodaphnia dubia*; rapid mortality; no pH effects on toxicity.

Invertebrates - Chronic Tests

- Norberg-King (1988) - Fifteen *C. dubia* tests conducted in three different dilution waters resulted in IC_{50}s with CV of 29 per cent.

- Taraldsen *et al.* (1989) - Consistent results obtained in *Ceriodaphnia dubia* tests (data not given); no hardness or pH effects on toxicity.

- Anderson *et al.* (1989) - Chronic *C. dubia* tests in ten laboratories resulted in IC_{50}s with CV of 29 per cent.

Algae Tests

- Taraldsen *et al.* (1989) - Reproducible results achievable using NaCl in algae tests (no data given); no pH or hardness dependency.

Marine Tests

- Inappropriate since one cannot use a saline solution to test marine organisms.

Microtox ™

- No data found.

A.2.2.6 Zinc ($ZnSO_4$ or $ZnCl_2$)

Fish - Acute Tests

- Bradley and Sprague (1985) - Three acute rainbow trout tests conducted at similar pH (7) and hardness (30 mg $CaCO_3$/L) gave mean LC_{50} of 0.16 mg Zn/L with CV of 27 per cent.

- Boyce and Yamada (1977) - Sockeye salmon infected with an intestinal parasite showed reduced tolerance (measured as LC_{50}) to zinc relative to non-infected fish.

- K. Doe, unpublished data - Nine acute lethality tests using rainbow trout and $ZnSO_4$ gave LC_{50}s with a CV of 19 per cent; three tests using the same species and $ZnCl_2$ gave LC_{50}s with a CV of 3.4 per cent; five tests using threespine sticklebacks and $ZnSO_4$ gave LC_{50}s with a CV of 7.8 per cent; zinc chloride formed precipitate in stock solution of 1 g/L and more in distilled water.

- See Table A.18.

Fish - Chronic Tests

- Spear (1981) - Reproductive inhibition and growth are sensitive indicators of zinc toxicity to fish.

Invertebrates - Acute Tests

- Thomas *et al.* (1986) - $ZnCl_2$ had little effect on *Daphnia sp.* in 48 hours at concentrations tested, but 72-hour EC_{50}s showed a CV of 30 per cent (N = 5).

Invertebrates - Chronic Tests

- No data found.

Algae Tests

- Thomas *et al.* (1986) - Five algae tests (no species given) gave a CV of 12 per cent (Cl_2).

- D. St.-Laurent, unpublished data - Three 96-hour microplate tests using *Selenastrum capricornutum* gave EC_{50}s with CV of 22 per cent ($ZnCl_2$) ($\bar{x}$ EC_{50} = 52 (µg/L).

Marine Tests

- No particular problems found to be associated specifically with tests conducted in marine rather than fresh waters.

- U.S. EPA (1980h) - Tests conducted with *Mytilus medilus* (N = 3), *Homarus americanus* (N = 4), and *Menidia menidia* (N = 5) gave LC_{50}s with CV of 26, 48, and 24 per cent, respectively ($ZnCl_2$).

Microtox ™

- Thomas *et al.* (1986) - Five Microtox tests conducted with $ZnCl_2$ gave 5-, 15-, and 30-minute EC_{50}s with CVs of 50, 45, and 35 per cent, respectively.

- Qureshi *et al.* (1982) - A single set of duplicate tests gave EC_{50}s with CV of 14 per cent.

Table A.1 Ranking[a] of Potential Reference Toxicants According to Primary Selection Criteria[b]

	Organic				Inorganic						
Criteria[c]	4-Chloro phenol	Dodecyl Sodium Sulphate (DSS)	Phenol	Sodium Pentachloro-phenate (NaPCP)	Cadmium ($CdCl_2$)	Chromium ($KCrO_4$) or ($K_2Cr_2O_7$)	Copper ($CuSO_4$)	Potassium Chloride (KCl)	Silver Nitrate ($AgNO_3$)	Sodium Chloride (NaCl)	Zinc ($ZnSO_4$)
Detection of Abnormal Organisms	0	no	yes	yes	0	E	no	0	0	E	yes
Established Toxicity Database	no	yes	yes	yes	yes	yes	yes	no	yes	yes	yes
Readily Available in Pure Form	yes	yes[g]	yes	yes	yes	yes	yes	yes	yes	yes	yes
Soluble	yes	yes[d]	yes	yes	yes	yes	yes[d]	yes	yes	yes	yes
Stable in Solution	yes	no	no	yes	yes	yes	yes[d]	yes	E	yes	yes
Stable Shelf Life	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Limited Intralaboratory Water Quality Effects	yes	yes	yes	E[e]	0[f]	yes[e,f]	yes[d,f]	yes	no	yes	yes[f]
Easily Analyzed	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
TOTAL SCORE	5	4	6	7	6	7	6	5	4	7	8

E = equivocal; conflicting reports
0 = too few data

[a] Addition of "yes" items and subtraction of "no" gives total; other symbols (0 and E) have no score
[b] Supporting data presented in Appendix Tables A.2 to A.12
[c] Complete description in text, Appendix A
[d] Not in some waters
[e] Some pH effects
[f] Some hardness effects
[g] Batches may vary in toxicity

Table A.2 4-Chlorophenol (*para*-Chlorophenol) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	No data	
Established Toxicity Database	No (U.S. EPA, 1980b)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 98 to 99%)	
Soluble	Slightly (Windholz *et al.*, 1983)	
Stable in Solution	(K. Doe, unpubl. data)	
Stable Shelf Life	Normally stable (BDH MSD sheet)	
Limited Water Quality Effects	(K. Doe, unpubl. data)	
Easily Analyzed	Gas Chromatography	

Table A.3 Dodecyl Sodium Sulphate (DSS) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms		A diseased stock of rainbow trout produced an LC_{50} similar to that for healthy fish (Pessah *et al., 1975).* Rainbow trout stressed by starvation, temperature or crowding responded similarly to DSS relative to control fish as did different strains of trout (median survival time) (Alexander and Clarke, 1978)
Established Toxicity Database	Pickering (1988) Lewis and Weber (1985) Weber *et al.* (1989) Fogels and Sprague (1977) Foy (1982)	
Readily Available in Pure Form	Canlab (95%) BDH (100%)	Toxicity may vary between batches (Fogels and Sprague, 1977)
Soluble	Windholz *et al.* (1983)	Poor solubility in cold, marine waters (Riebel, 1988)
Stable in Solution		Degrades (Pessah *et al.*, 1975; Abel, 1974)
Stable Shelf Life	Toxicity of batch obtained in early 1970s has remained consistent (K. Doe, unpubl. data)	
Limited Water Quality Effects	Karande and Goankar (1987)	
Easily Analyzed	Gas Chromatography	

Table A.4 Phenol Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	Phenol assays showed differences between unstressed fish and fish stressed by starvation, temperature and chlorine exposure; some differences between genetic strains observed; no difference resulted from crowding stress or elevated holding mortality (Alexander and Clarke, 1978). Nutritional deficiency detected in trout stock over time (J. Somers, unpubl. data)	
Established Toxicity Database	U.S. EPA (1980f), Milleman *et al.* 1984), Buikema *et al.* (1979)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 99%)	
Soluble	Approximately 67 g/L (Windholz *et al.*, 1983)	
Stable in Solution		Degrades (Buikema *et al.*, 1979)
Stable Shelf Life	Except when exposed to air or light (BDH MSD sheet)	
Limited Water Quality Effects	Only minimal effects from pH and hardness (Fogels and Sprague, 1977; Dalela *et al.*, 1980)	
Easily Analyzed	4-AAP	

Table A.5 Sodium Pentachlorophenate (NaPCP) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	Rainbow trout exposed to starvation or temperature-stress showed difference in median survival time from normal fish; temperature-stressed fish showed no difference; no difference between genetic strains (Alexander and Clarke, 1978). Unusual response (low LC_{50}) of one batch of rainbow trout attributed to smaller size; same fish showed decreasing LC_{50} and decreasing condition factor over 4 weekly tests (Hansen *et al.*, 1979)	
Established Toxicity Database	U.S. EPA (1980e), McKee *et al.* (1984), DeGraeve *et al.* (1989), Weber *et al.* (1989)	
Readily Available in Pure Form	Aldrich (93%)	
Soluble	McKee *et al.* (1984)	
Stable in Solution	Lee (1980)	
Stable Shelf Life	BEAK (unpubl. data)	
Limited Water Quality Effects	Dilution water pH ranging from 7.5 to 8.2 resulted in coefficients of variation of 14 to 31% for rainbow trout (acute LC_{50}), *Daphnia magna* (acute LC_{50}), fathead minnows (chronic LC_{50} and IC_{50}) and *Ceriodaphnia* (chronic LC_{50} and IC_{50}) (BEAK, unpubl. data)	pH shifts during tests (Degraeve *et al.*, 1989)
Easily Analyzed	Gas Chromatography - Electron Capture Detector	

Table A.6 Cadmium Chloride ($CdCl_2$) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	No data	
Established Toxicity Database	U.S. EPA (1980a), Lewis and Weber (1985), Weber *et al.* (1989)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 99%)	
Soluble	Freely soluble (Windholz *et al.*, 1983)	
Stable in Solution	G. Callous (pers. comm.)	
Stable Shelf Life	BDH, MSD sheet	
Limited Water Quality Effects	McCracken (1987), Sprague (1987)	
Easily Analyzed	DCP	

Table A.7 Hexavalent Chromium (as $K_2Cr_2O_7$ or $KCrO_4$) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	High control mortality in *Daphnia pulex* and *Mysidopsis bahia* acute tests correlated with greater sample toxicity and attributed to unhealthy culture/stocks (Dorn and Rodger, 1989)	Unhealthy goldfish stock (high holding mortality) gave similar LC_{50} result to healthy fish (Adelman and Smith, 1976)
Established Toxicity Database	U.S. EPA (1980c), Jop *et al.* (1986, 1987), DeGraeve *et al.* (1989), CCREM (1987)	
Readily Available in Pure Form	BDH, Canlab, Fisher (99-100%)	
Soluble	e.g., 4.3% at 20°C for $K_2Cr_2O_7$ (Windholz *et al.*, 1983)	
Stable in Solution	Yes (Jop *et al.*, 1986)	
Stable Shelf Life	Normally stable (MSD sheet)	
Limited Water Quality Effects	Jop *et al.* (1987) (although some pH effects may result; this probably minimized by appropriate use of either CrO_4 or Cr_2O_7 {Section 2.0})	
Easily Analyzed	Colourimetric or Atomic Absorption Spectrometry	

Table A.8 Copper Sulphate ($CuSO_4$) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms		Rainbow trout stressed by starvation or crowding did not show significant difference from normal fish; also no difference between genetic strains (Alexander and Clarke, 1978)
Established Toxicity Database	U.S. EPA (1980d)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 97 to 99%)	
Soluble	Very (Windholz *et al.*, 1983)	
Stable in Solution	Stable in water supplies that do not contain high concentrations of complexing agents (see right)	Cupric ion is highly reactive and may complex and precipitate with other aqueous constituents such as carbonate, phosphate, humates, and suspended solids (U.S. EPA, 1980d). Increased complexation occurs with increased salinity. Colloidal dispersion or precipitates may occur in marine waters (Spear and Pierce, 1979)
Stable Shelf Life	Except when exposed to heat or air (BDH MSD sheet)	
Limited Water Quality Effects		
Easily Analyzed	DCP	

Table A.9 Potassium Chloride (KCl) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	No data	
Established Toxicity Database	No	
Readily Available in Pure Form	BDH, Canlab, Fisher	
Soluble	Windholz *et al.* (1983)	
Stable in Solution		
Stable Shelf Life	Yes (Mallinckrodt, MSDS)	
Limited Water Quality Effects	G. Callous, B. Peltier (pers. comm.)	
Easily Analyzed	Ion chromatography	

Table A.10 Silver Nitrate ($AgNO_3$) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	No data	
Established Toxicity Database	U.S. EPA (1980g)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 99.9%)	
Soluble	2500 g/L (Windholz *et al.*, 1983)	
Stable in Solution	Measured concentrations similar to nominal (Lemke, 1981)	Reacts readily with other aqueous constituents (e.g., chlorides) (Fisher, MSD sheet); stripped by heavy aeration (Lemke, 1981)
Stable Shelf Life	Normally stable (Fisher MSD sheets)	
Limited Water Quality Effects		Highly affected by hardness (Lemke, 1981), salinity (Dinnel, 1982, 1987)
Easily Analyzed	DCP	

Table A.11 Sodium Chloride (NaCl) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	LC_{50} of one unhealthy gold fish stock (high mortality) was out of range of results for healthy fish (Adelman and Smith, 1976 and Adelman *et al.*, 1976; same data in both studies)	A batch of diseased (fungus) rainbow trout gave LC_{50} that fell into normal range (T. Kovacs, pers.comm.); consistent results were obtained with fish that showed high holding mortality (golden shiners and sticklebacks). Rainbow trout that were smaller and had low condition factor also produced LC_{50}s consistent with other stocks (Hansen *et al.*, 1979)
Established Toxicity Database	Taraldsen *et al.* (1989) Norberg-King (1988) Anderson *et al.* (1989) Adelman & Smith (1976)	Lacks published review data
Readily Available in Pure Form	Canlab, Fisher, BDH (greater than 99%)	
Soluble	Windholz *et al.* (1983)	
Stable in Solution	Yes	
Stable Shelf Life	Mallinckrodt MSD sheet	
Limited Water Quality Effects	Taraldsen *et al.* (1989) T. Norberg-King (pers. comm.)	
Easily Analyzed	Ion chromatography, conductivity, titrimetry	

Table A.12 Zinc ($ZnSO_4$ or $ZnCl_2$) Supporting Documentation

Criteria	Yes	No
Detection of Abnormal Organisms	Sockeye salmon infected with an intestinal parasite showed reduced tolerance (measured as LC_{50}) to zinc relative to non-infected fish (Boyce & Yamada, 1977)	
Established Toxicity Database	U.S. EPA (1980h) CCREM (1987)	
Readily Available in Pure Form	BDH, Canlab, Fisher (e.g., 99%)	
Soluble	$ZnCl_2$ - 432 g/100 g water; $ZnSO_4$ - 167 g/100 g water (Windholz *et al.*, 1983)	$ZnCl_2$ has given precipitate in distilled water at concentrations greater than 1 g/L (K. Doe, unpubl. data)
Stable in Solution	Yes	
Stable Shelf Life	BDH MSD sheet	
Limited Water Quality Effects	Hardness, alkalinity and pH affect toxicity (EPA, 1980h), but extent of effects on test reproducibility not known	
Easily Analyzed	DCP	

Table A.13 Reproducibility of Results Using Specific Reference Toxicants in Specific Test Types

	Organic			Inorganic					
Test Type	4-Chloro-phenol	Phenol[b]	Sodium Pentachloro-phenate (NaPCP)[c]	Cadmium ($CdCl_2$)	Hexavalent Chromium (as $K_2Cr_2O_7$ or K_2CrO_4)	Copper ($CuSO_4$)	Potassium Chloride (KCl)	Sodium Chloride (NaCl)	Zinc ($ZnSO_4$ or $ZnCl_2$)
Fish:									
Acute	yes	yes	yes	no	yes[d]	yes[e]	?	yes[h]	yes
Chronic	?	?	yes	no	yes[d]	yes[e]	?	?	yes
Invertebrates:									
Acute	yes	yes[g]	yes	no	yes	?	?	?	yes
Chronic	?	?	yes	?	?	?	?	yes	?
Algae	?	yes	?	no	yes	yes[e]	?	?	yes
Marine Tests	yes	yes	yes	no[f]	yes	no	?	no	yes
Microtox ™	?	yes	yes	no	?	no	?	no	no

[a] Table is based on data presented in Sections A.2.1 to A.2.9.
[b] Some problems reported attributable to bacterial degradation.
[c] Some problems reported attributable to pH.
[d] Some problems reported related to variable responses in fish tests.
[e] Not untreated natural supplies which may contain variable water quality (e.g., hardness, pH) or complexing agents.
[f] "no" is a result of unacceptable repeatability in organism responses, rather than incompatibility with marine waters.
[g] Possibly unacceptable declines in dissolved oxygen levels in unaerated tests.
[h] Probably not best choice for species tolerant of saltwater (e.g., rainbow trout).
? No data available, or conflicting data.

Table A.14 Acute Toxicity of Copper to Fish (from U.S. EPA, 1980d)*

Test Organism						Hardness Range	Copper	
Scientific Name	Common Name	N	$\bar{x}$ (µg/L)	SD	%CV	(mg $CaCO_3$/L)	Species	Comments
Oncorhynchus kisutch	coho salmon smolt	3	68	7.2	11	89-99	Chloride	
Oncorhynchus tschawytscha	chinook salmon (alevin to smolt)	4	27	7.9	29	25	?	
(Oncorhynchus mykiss) (formerly *Salmo gairdneri*)	rainbow trout	5	31	0.84	2.7	30-32	Sulphate	
		8	52	21	40	98-102	Sulphate	Over range of pH
		10	198	122	62	194	Chloride	Over range of pH
		4	23	6.4	28	25	?	
		3	200	10	5	125	Sulphate	Body size 3.9 g - 176 g
Pimephales notatus	bluntnose minnow	5	286	33	12	200	Sulphate	
		3	233	32	14	194	Sulphate	
Pimephales promelas	fathead minnow	3	108	17	16	45-48	?	
Lepomis gibbosus	pumpkinseed	7	1541	280	18	125	Sulphate	
Trachinotus carolinus	Florida pompano	3	417	81	20	Marine	Sulphate	

* Studies in which three or more tests were conducted within a limited range of water hardness level.

Table A.15 Chronic Toxicity of Copper to Fish (U.S. EPA, 1989a)

Test Organisms						
Scientific Name	Common Name	Water Quality	Test Type	End Point	Values (µg/L)	N
Pimephales promelas	Fathead minnow	Fresh, hardness = 180 mg $CaCO_3$/L	7-d* Larval Survival and Growth	NOEC	25-25	2
				LOEC	25-50	2
Cyprinodon variegatus	Sheepshead minnow	Natural seawater, 20 ppt	7-d Larval Survival and Growth	NOEC (Survival)	125-250	5
				LOEC (Survival)	250-500	5
				NOEC (Growth)	31-125	5
				LOEC (Growth)	63-250	5
		Artificial seawater, FORTY FATHOMS, 20 ppt	7-d Larval Survival and Growth	NOEC (Survival)	50-100	8
				LOEC (Survival)	100-200	8
				NOEC (Growth)	50	8
				LOEC (Growth)	100	8
		Artificial seawater, HW MARINEMIX, 20 ppt	9-d Embryo-Larval Survival and Teratogenicity	NOEC	200-240	8
				LOEC	220-270	8
Menidia beryllina	Inland silverside	Natural seawater 30 ppt	7-d Larval Survival and Growth	NOEC (Survival)	63-125	5

* Day

Table A.16 Acute Toxicity of Copper to Invertebrates

Test Organism		N	$\bar{x}$	SD	%CV	LC_{50} Range	Hardness		
Scientific Name	Common Name		(µg/L)			(µg/L)	(mg $CaCO_3$/L)	Comment	Source
Daphnia pulicaria	Cladoceran	5	9.6	1.6	17	7.2-11	44-48		from U.S. EPA, 1980d
Arcantia tonsa	Calenoid copepod	3	34	19	57	17-55	-		from U.S. EPA, 1980d
Daphnia magna	Cladoceran	13				6.5-200	-	Results may have been affected by water hardness and pH effects	from U.S. EPA, 1989a

Table A.17 Chronic and Sublethal Toxicity of Copper to Invertebrates (U.S. EPA, 1989a)

Test Organisms					Values	
Scientific Name	Common Name	Water Quality	Test Type	End Point	(µg/L)	N
Daphnia magna	Cladoceran	Freshwater	Chronic Survival and Reproduction	Chronic Value	9.5-29.3	3
Mysidopsis bahia	Mysid shrimp	Natural Seawater, 30 ppt	7-d Survival, Growth and Fecundity	NOEC (Survival)	63-125	6
				LOEC (Survival)	125-250	6
		Artificial Seawater FORTY FATHOMS, 30 ppt	7-d Survival, Growth and Fecundity	NOEC (Survival)	75	5
				LOEC (Survival)	150	5
				NOEC (Growth)[a]	9-75	5
				LOEC (Growth)[a]	38-150	5
				NOEC (Fecundity)[b]	38-75	5
				LOEC (Fecundity)[b]	75-150	5
Arbacia punctulata	Sea urchin	Natural Seawater, 30 ppt	Fertilization	NOEC	6.1-24.4	5
				LOEC	6.1-48.7	5
		Artificial Seawater, FORTY FATHOMS, 30 ppt	Fertilization	NOEC	5.0-12.5	5
				LOEC	6.2-25.0	5

[a] Copper had no statistically significant effect on growth over a range of 9 to 150 µg/L in two of the five tests.

[b] Copper had no statistically significant effect on fecundity over a range of 9 to 150 µg/L in three of the five tests.

Table A.18 Acute Toxicity of Zinc to Fish (U.S. EPA, 1980h)

Test Organism	N	$\bar{x}$ (µg/L)	SD	%CV	Hardness (mg $CaCO_3$/L)	Zinc Species
Salmo gairdneri *	5	570	255	45	30	Sulphate
S. gairdneri	3	548	195	36	44-47	Sulphate
S. gairdneri		2 460	527	21	170-179	Sulphate
Salvelinus fontinalis	3	2 030	442	22	44-47	Sulphate
S. fontinalis	3	6 033	1004	17	170-179	Sulphate
Pimephales promelas	4	10 850	2055	19	203	Sulphate
Lepomis macrochirus	4	5 375	400	7.4	20	Sulphate

* *Salmo gairdneri* (Rainbow Trout) is now known as *Oncorhynchus mykiss*

Appendix B

Health and Safety Data

B.1 Data Sources

Health and safety data associated with each of the chemicals identified as suitable toxicants were obtained from the following sources:

- Material Safety Data Sheets
  -BDH Inc.
  -Fisher Scientific
  -Canlab, A Division of Travenol Canada Inc.
  -Aldrich Ltd.
- Sax and Lewis, 1987
- Windholz *et al.*, 1983

B.2 Disposal of Test Solutions

Regulations regarding disposal of solutions to municipal sewers will vary widely between municipalities. All laboratories should consult their local municipal office prior to discharging chemical waste to the sewer system. Frequently the regulations are concentration-based and, if the highest test concentration does not exceed the regulation limit, the solutions can be disposed of down the drain.

If any test solutions exceed a regulation limit, then the method of disposal must be reconsidered. It may be possible to combine the more dilute solutions from a test with those that are more concentrated to achieve levels below the regulated limit. Dilution by running water from the tap is discouraged. Temporary retention of all laboratory wastewaters in an equalization tank prior to discharge to the sewer system may achieve acceptable concentrations since the chemical solution is mixed with other dilute wastewaters from the laboratory.

Some chemicals may require disposal in proper containers to be hauled away by licensed waste disposal companies. Selection of an alternative reference toxicant, however, may be an easier, less expensive alternative.

Table B.1 4-Chlorophenol (4-ClC_6H_4OH) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid	Specific Gravity (kg/L):	1.306 (20°C)
Boiling Range (°C):	220	Freezing Point (°C):	41-43
Molecular Weight:	128.56	Vapour Pressure:	1 mm Hg at 50°C
Water Solubility:	2.7 parts per 100 parts at 20°C	pH:	Not available.
Evaporation Rate:	Not available.	Viscosity cSt @ 40°C:	Not applicable.
Odour and Appearance:	Chrystalline solid; phenolic odour.		

FIRE AND EXPLOSION DATA

Flash Point (COC °C):	121°C/cc	Auto Ignition Temp. (°C):	Not available.
Expl. Limit Lower (%):	Not available.	Expl. Limit Upper (%):	Not available.
Extinguishing Media:	Dry chemical; foam; water spray (fog); avoid water because of pollution potential.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Toxic; destructive to mucous membranes.
Skin Contact:	Causes burns; absorbed through skin; toxic.
Eye Contact:	Causes burns.
Ingestion:	Toxic.

Emergency and First Aid Procedures

Inhalation:	Move victim to fresh air. If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Ingestion:	Rinse mouth thoroughly with water. Do not induce vomiting. Have victim drink 200-400 mL of water to dilute. If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.

Additional Toxicology Information

LD_{50}: 500 mg/kg (oral, rat); possibly not carcinogenic; possibly mutagenic; skin irritation (rabbit): 2 mg/24 h severe; LC_{50}: 11 mg/m^3 (inhalation, rat).

REACTIVITY DATA

Chemical Stability:	Stable; may be sensitive to light and moisture.
Incompatability (conditions to avoid):	Oxidizing materials, bases.
Hazardous Decomposing Products:	Hydrochloric acid, CO_2, chlorine compounds.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Dust mask, self-contained breathing apparatus.
Eyes:	Goggles or faceshield.
Gloves:	Nitrile.
Other:	Plastic apron, sleeves and boots, as appropriate.
Engineering Controls (i.e., ventilation, enclosed process):	Mechanical ventilation.
Leak and Spill Procedures:	Take protective measures. Shut off all possible sources of ignition. Mix with sand, transfer carefully into container and arrange for disposal.
Waste Disposal:	Follow all federal, provincial, and local regulations; see Section B.2
Handling Procedures and Equipment:	Follow all safe handling procedures.
Storage Requirements:	Suitable labelled, tightly closed containers. Store in a cool, dry, well-ventilated area out of direct sunlight. Keep away from ignition sources.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.2 Phenol (C_6H_5OH) Health and Safety Data

PHYSICAL DATA FOR MATERIAL			
Physical State:	Solid	Specific Gravity (kg/L):	Not available
Boiling Range (°C):	182	Freezing Point (°C):	41-43
Molecular Weight:	94.11	Vapour Pressure:	1.0 mmHg at 40.1°C
Water Solubility:	1 g in 15 mL	pH:	6.0
Odour and Appearance:	Colourless to pink with a characteristic sweet, tarry odour.		

FIRE AND EXPLOSION DATA			
Flash Point (COC °C):	79	Auto Ignition Temp. (°C):	715
Expl. Limit Lower (%):	3.0	Expl. Limit Upper (%):	10.0
Extinguishing Media:	Dry powder or vaporizing liquids, carbon dioxide, foam.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation: Irritation of nose and throat, nausea, vomiting and diarrhea.

Skin Contact: Corrosive, risk of absorption is high, can cause central nervous system effects (nausea, dizziness, headache) and possible liver and kidney damage.

Eye Contact: Irritation; can cause welling and clouding of cornea.

Ingestion: Severe burns, abdominal pain, cyanosis, muscular weakness, tremor, and convulsions.

Emergency and First Aid Procedures

Inhalation: Remove from exposure; rest and keep warm. Seek medical attention.

Skin Contact: Swab contaminated skin with glycerol, polyethylene glycol 300. Flush with water. Seek medical attention.

Eye Contact: Flush with water for at least 10 minutes. Seek medical attention.

Ingestion: Wash out mouth thoroughly and give water to drink. Seek medical attention.

Additional Toxicology Information

LD_{50}: 317 mg/kg (oral, rat); maximum acceptable air exposure is 19 mg/m^3 as time weighted average; maximum dermal exposure is 5 ppm as time weighted average; probably not carcinogenic.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Strong oxidizing agents, mixture with aluminum chloride and nitrobenzene; slowly turns pink or red when exposed to air or liquid.
Hazardous Decomposing Products:	None

PREVENTIVE MEASURES (Personal Protective Equipment)

Respiratory:	Self-contained breathing apparatus.
Eyes:	Goggles or faceshield.
Gloves:	Nitrile.
Other:	Plastic apron, sleeves or boots, as appropriate.
Engineering Controls (i.e., ventilation, enclosed process):	Local exhaust ventilation, general ventilation may be adequate for small-scale use at room temperature.
Leak and Spill Procedures:	Mix with sand, transfer carefully into containers; wash site thoroughly with water and detergent.
Waste Disposal:	Follow all federal, provincial, and local regulations; see Section B.2.
Handling Procedures and Equipment:	Keep away from sources of heat or flame. Follow routine safe handling procedures.
Storage Requirements:	Store in tightly sealed containers in a cool, dry place. Protect from exposure to sunlight.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.3 Sodium Pentachlorophenate (C_6HCl_5ONa) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid	Specific Gravity (kg/L):	1.978
Boiling Range (°C):	310	Melting Point (°C):	190
Molecular Weight:	Not available.	Vapour Pressure:	40 mm Hg @ 211.2°C
Water Solubility:	8 mg/100 mL	pH:	Not available.
Evaporation Rate:	Not applicable.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	Beige powder or crystals; pungent odour when hot.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not available.	Auto Ignition Temp. (°C):	Not available.
Expl. Limit Lower (%):	Not available.	Expl. Limit Upper (%):	Not available.
Extinguishing Media:	Carbon dioxide; dry chemical; foam; water spray; emits toxic fumes under fire conditions		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	May be fatal; nausea, dizziness, headache.
Skin Contact:	Irritation; absorption may be fatal.
Eye Contact:	Irritation.
Ingestion:	May be fatal; nausea, dizziness, headache.

Emergency and First Aid Procedures

Inhalation:	Remove to fresh air. If breathing has stopped, begin AR. Seek medical attention.
Skin Contact:	Flush with copious amounts of water for at least 15 minutes.
Eye Contact:	Flush with copious amounts of water for at least 15 minutes.
Ingestion:	Induce vomiting immediately.

Additional Toxicology Information

LD_{50}:50: mg/kg (oral, rat); LD_{50}: 11.7 mg/kg (inhalation, rat); maximum acceptable air exposure 0.5 mg/m^3 as time weighted average; permeable through skin; possible teratogen, carcinogenic.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Strong oxidizing agents.
Hazardous Decomposing Products:	Toxic fumes of carbon monoxide, carbon dioxide, hydrogen chloride gas.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Approved respirator.
Eyes:	Safety goggles.
Gloves:	Rubber.
Other:	Protective clothing.
Engineering Controls (i.e., ventilation, enclosed process):	Local or general exhaust.
Leak and Spill Procedures:	Wear self-contained breathing apparatus, rubber boots, and heavy rubber gloves. Sweep up, place in a bag; arrange for disposal. Wash spill site.
Waste Disposal:	Follow all federal, provincial, and local regulations; see Section B.2.
Handling Procedures and Equipment:	Follow routine safe handling procedures.
Storage Requirements:	Keep in tightly closed container. Store in a cool, dry place.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.4 Cadmium Chloride ($CdCl_2$) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	4.000
Boiling Range (°C):	960	Melting Point (°C):	568
Molecular Weight:	183.32	Vapour Pressure:	10 mm Hg at 656 C
Water Solubility:	Freely Soluble.	pH:	4.0-6.5 (5% solution)
Evaporation Rate:	Not available.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	White, odourless crystals.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Use an extinguisher appropriate to the surrounding material which is burning.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Harmful: chronic exposure - lung and kidney damage, renal and hepatic deterioration; acute exposure - nausea, diarrhea, abdominal pain and choking.
Skin Contact:	Irritation.
Eye Contact:	Irritation.
Ingestion:	Harmful - same as inhalation.

Emergency and First Aid Procedures

Inhalation:	If not breathing, begin AR; if heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Ingestion:	Rinse mouth, drink 200-400 mL water - induce vomiting. Seek medical attention.

Additional Toxicology Information

LD_{50}: 88 mg/kg (oral, rat); maximum acceptable air exposure is 200 $\mu g/m^3$ as a time weighted average; carcinogenic; may be mutagenic and teratogenic.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	BF3, potassium, strong oxidizers, acids.
Hazardous Decomposing Products:	Cadmium oxides, chlorine compounds.

PREVENTIVE MEASURES (Personal Protective Equipment)

Respiratory:	Dust mask.
Eyes:	Chemical safety goggles.
Gloves:	Rubber or plastic.
Other:	Plastic apron and boots, as appropriate.
Engineering Controls (i.e., ventilation, enclosed process):	Mechanical ventilation.
Leak and Spill Procedures:	Shut off all possible sources of ignition. Transfer carefully into container and arrange removal. Wash site with water and detergent.
Waste Disposal:	Follow federal, provincial, and local regulations; see Section B.2.
Handling Procedures and Equipment:	Avoid generating dust. Follow routine safe handling procedures.
Storage Requirements:	Labelled containers, tightly closed, protect from damage.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.5 Potassium Dichromate ($K_2Cr_2O_7$) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	2.68
Boiling Range (°C):	500	Freezing Point (°C):	398
Molecular Weight:	294.18	Vapour Pressure:	Not available.
Water Solubility:	Soluble	pH:	3.57
Evaporation Rate:	Not applicable.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	Bright orange red crystals, odourless.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Use an extinguisher appropriate to the surrounding material which is burning.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Harmful dust; may cause perforation of nasal septum.
Skin Contact:	Corrosive; absorption harmful; may cause rash or external ulcers.
Eye Contact:	Corrosive.
Ingestion:	Harmful - stomach and kidney disorders.

Emergency and First Aid Procedures

Inhalation:	Move victim to fresh air; if not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush the area with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush eyes for at least 15 minutes with lukewarm running water. Seek medical attention.
Ingestion:	Rinse mouth with water; do not induce vomiting. Have victim drink 200-400 mL water to dilute. If breathing has stopped, begin AR; if heart has stopped, begin CPR. Seek medical attention.

Additional Toxicology Information

LD_{50}: 190 mg/kg (oral, mouse); carcinogen.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Anhydrous hydroxylamine, organic materials, reducing agents; excessive heat causes decomposition.
Hazardous Decomposing Products:	Chromium oxides.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Dust mask, self-contained breathing apparatus.
Eyes:	Chemical safety goggles or faceshield.
Gloves:	Rubber, plastic or nitrile.
Other:	Plastic apron, sleeves and boots, as appropriate.
Engineering Controls (i.e., ventilation, enclosed process):	Mechanical ventilation.
Leak and Spill Procedures:	Shut off all possible sources of ignition. Transfer spillage into containers of water or mix with wet sand and carefully transfer to containers.
Waste Disposal:	Follow all federal, provincial and local regulations; see Section B.2.
Handling Procedures and Equipment:	Avoid generating dust. Follow routine safe handling procedures.
Storage Requirements:	Store in suitable labelled, tightly closed containers. Store in a cool, dry, well-ventilated area away from materials which can burn.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.6 Potassium Chromate (K_2CrO_4) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	2.73
Boiling Range (°C):	Not available.	Melting Point (°C):	975
Molecular Weight:	194.19	Vapour Pressure:	Not available.
Water Solubility:	Soluble in 1.6 parts cold water.	pH:	Not available.
Evaporation Rate:	Not applicable.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	Yellow solid, odourless.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Carbon dioxide, dry chemical, foam.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Irritates upper respiratory tract.
Skin Contact:	Irritates; absorption harmful; may cause rash or external ulcers.
Eye Contact:	Irritates.
Ingestion:	Harmful, may cause vomiting, diarrhea, nephritis, liver damage and dermatitis.

Emergency and First Aid Procedures

Inhalation:	Move victim to fresh air; if not breathing, begin AR; if heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush the area with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Ingestion:	Rinse mouth thoroughly with water. Do not induce vomiting. Have victim drink 200-400 mL of water to dilute. If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.

Additional Toxicology Information

LD_{50}: 50 mg/kg (oral, human); maximum acceptable air exposure is 100 $\mu g/m^3$ as a time weighted average; carcinogen.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Reducing agents, acids and organic materials.
Hazardous Decomposing Products:	Chromium oxides.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Self-contained breathing apparatus.
Eyes:	Chemical safety goggles.
Gloves:	Rubber or plastic.
Other:	Plastic apron, sleeves and boots, as appropriate.

Engineering Controls (i.e., ventilation, enclosed process):	Mechanical ventilation.
Leak and Spill Procedures:	Carefully transfer to container of large volume of water. Add an excess of sodium hypochlorite and allow to stand for 24 hours.
Waste Disposal:	Follow all federal, provincial and local regulations; see Section B.2.
Handling Procedures and Equipment:	Avoid generating dust. Follow routine safe handling procedures.
Storage Requirements:	Suitable labelled containers. Store in cool, dry, well-ventilated area out of direct sunlight. Protect from damage.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.7 Cupric Sulphate ($CuSO_4$) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	**Specific Gravity (kg/L):**	3.6
Boiling Range (°C):	150	**Freezing Point (°C):**	200
Molecular Weight:	159.60	**Vapour Pressure:**	Negligible .
Water Solubility:	Soluble.	**pH:**	Not available.
Evaporation Rate:	Not applicable.	**Viscosity cSt @ 40°C:**	Not applicable.
Odour & Appearance:	Grayish-white odourless powde..		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	**Auto Ignition Temp. (°C):**	Not applicable.
Expl. Limit Lower (%):	Not applicable.	**Expl. Limit Upper (%):**	Not applicable.
Extinguishing Media:	Use an extinguisher appropriate to the surrounding burning material.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Toxic by dust inhalation.
Skin Contact:	Severe irritation - slight absorption may occur.
Eye Contact:	Severe irritation.
Ingestion:	Toxic.

Emergency and First Aid Procedures

Inhalation:	If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Ingestion:	Rinse mouth thoroughly with water. Do not induce vomiting. Have victim drink 200-400 mL of water to dilute. If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.

Additional Toxicology Information

LD_{50}: 300 mg/kg (oral, rat); maximum acceptable air exposure is 1 mg/m^3 as time weighted average.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Very hygroscopic with generation of heat. May slowly oxidize on exposure to air.
Hazardous Decomposing Products:	Toxic sulphur oxide gases at high temperatures.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Self-contained breathing apparatus.
Eyes:	Goggles or faceshield.
Gloves:	Rubber or plastic.
Other:	Plastic apron, sleeves and boots, as appropriate.

Engineering Controls (i.e., Ventilation, Enclosed Process):	Mechanical ventilation.
Leak and Spill Procedures:	Shut off all possible sources of ignition; mop up with plenty of water or transfer to containers and arrange for disposal. Wash site thoroughly with water and detergent.
Waste Disposal:	Follow all federal, provincial, and local regulations; see Section B.2.
Handling Procedures and Equipment:	Handle under nitrogen. Follow routine safe handling procedures.
Storage Requirements:	Store under nitrogen or in dessicator in tightly closed container in a cool, dry place.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.8 Potassium Chloride (KCl) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	1.984
Boiling Range (°C):	1500 (sublimes)	Melting Point (°C):	772
Molecular Weight:	74.55	Vapour Pressure:	Not available.
Water Solubility:	35.7 g/100 g	pH:	7
Evaporation Rate:	Not applicable.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	White or colourless, odourless crystals.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Use any means suitable for extinguishing surrounding fire.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	High concentrations may cause nasal or lung irritation.
Skin Contact:	Irritation or rash, particularly with moist skin.
Eye Contact:	Irritation - possible abrasion.
Ingestion:	Large quantities can produce gastrointestinal irritation and vomiting.

Emergency and First Aid Procedures

Inhalation:	Remove to fresh air; seek medical attention for any breathing difficulty.
Skin Contact:	Wash with running water for at least 15 minutes.
Eye Contact:	Wash with running water for at least 15 minutes; seek medical attention.
Ingestion:	Give several glasses of water; seek medical attention.

Additional Toxicology Information

LD_{50}: 3020 mg/kg.

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Bromine trifluoride, potassium permanganate plus sulphuric acid.
Hazardous Decomposing Products:	Oxides of the contained metal and halogen, possibly also free or ionic halogen.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Dust mask.
Eyes:	Chemical safety goggles.
Gloves:	Protective.
Other:	Clean, body-covering clothing.
Engineering Controls (i.e., ventilation, enclosed process):	Local exhaust system.
Leak and Spill Procedures:	Sweep, scoop or pick up spilled material. Transfer to a closed preferably metal container.
Waste Disposal:	Follow all federal, provincial and local regulations; see Section B.2.
Handling Procedures and Equipment:	Follow routine safe handling procedures.
Storage Requirements:	Tightly closed containers; cool, dry, well-ventilated area; protect from physical damage.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.9 Sodium Chloride (NaCl) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	2.16
Boiling Range (°C):	1413	Melting Point (°C):	801
Molecular Weight:	58.44	Vapour Pressure:	1.0 @ 865°C
Water Solubility:	36 g/100 cc @ 20°C	pH:	6.7-7.3
Evaporation Rate:	Not available.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	White, odourless crystals.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Use an extinguisher appropriate to the surrounding material that is burning.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	Mild irritation to mucous membranes, nose and throat; coughing and sore throat.
Skin Contact:	Not expected to be a health hazard.
Eye Contact:	Irritation.
Ingestion:	Large doses can cause vomiting, diarrhea and prostration-dehydration and congestion in most internal organs.

Emergency and First Aid Procedures

Inhalation:	Move victim to fresh air. Seek medical attention for breathing difficulty.
Skin Contact:	Wash area with soap and water.
Eye Contact:	Wash thoroughly with running water. Seek medical attention.
Ingestion:	Seek medical attention.

Additional Toxicology Information

LD_{50}: 3000 mg/kg (oral, rat).

REACTIVITY DATA

Chemical Stability:	Stable.
Incompatability (conditions to avoid):	Lithium, bromide trifluoride.
Hazardous Decomposing Products:	When heated above 801°C, emits toxic fumes of chloride and sodium oxide.

PREVENTIVE MEASURES
(Personal Protective Equipment)

Respiratory:	Dust mask.
Eyes:	Chemical safety goggles.
Gloves:	Protective.
Other:	Clean, body-covering clothing.
Engineering Controls (i.e., ventilation, enclosed process):	Dilution ventilation.
Leak and Spill Procedures:	Sweep up and containerize.
Waste Disposal:	Follow all federal, provincial and local regulations; see Section B.2.
Handling Procedures and Equipment:	Follow routine safe handling procedures.
Storage Requirements:	Tightly closed containers; cool, dry, well-ventilated area; protect from physical damage.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Table B.10 Zinc Sulphate Heptahydrate ($ZnSO_4$ $7H_2O$) Health and Safety Data

PHYSICAL DATA FOR MATERIAL

Physical State:	Solid.	Specific Gravity (kg/L):	1.96
Boiling Range (°C):	Not available.	Melting Point (°C):	100
Molecular Weight:	287.54	Vapour Pressure:	Not available.
Water Solubility:	1 g/0.6 mL	pH:	4.5
Evaporation Rate:	Not applicable.	Viscosity cSt @ 40°C:	Not applicable.
Odour & Appearance:	White, odourless crystals or powder.		

FIRE AND EXPLOSION DATA

Flash Point (COC°C):	Not applicable.	Auto Ignition Temp. (°C):	Not applicable.
Expl. Limit Lower (%):	Not applicable.	Expl. Limit Upper (%):	Not applicable.
Extinguishing Media:	Non-flammable, use an extinguisher appropriate to the surrounding material that is burning.		

HEALTH HAZARD/FIRST AID PROCEDURE

Effects of Exposure

Inhalation:	May cause irritation.
Skin Contact:	May cause irritation; absorption may be harmful.
Eye Contact:	May cause irritation.
Ingestion:	May be harmful.

Emergency and First Aid Procedures

Inhalation:	Move victim to fresh air. If breathing has stopped, begin AR. If heart has stopped, begin CPR. Seek medical attention.
Skin Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Eye Contact:	Flush with lukewarm running water for at least 15 minutes. Seek medical attention.
Ingestion:	Rinse mouth thoroughly with water. Do not induce vomiting. Have victim drink 200-400 mL water to dilute. If not breathing, begin AR. If heart has stopped, begin CPR. Seek medical attention.

Additional Toxicology Information

LD_{50}: 2200 mg/kg (oral, rat); maximum exposure of 1 mg/m^3 in air as time weighted average.

REACTIVITY DATA

Chemical Stability:	Efflorescent in dry air; stable.
Incompatability (conditions to avoid):	Strong oxidizing agents.
Hazardous Decomposing Products:	Oxides of zinc.

PREVENTIVE MEASURES (Personal Protective Equipment)

Respiratory:	Dust mask; self-contained breathing apparatus.
Eyes:	Chemical safety goggles or face shield.
Gloves:	Rubber or plastic.
Other:	Plastic apron, sleeves and boots, as appropriate.
Engineering Controls (i.e., ventilation, enclosed process):	Mechanical ventilation.
Leak and Spill Procedures:	Take protective measures. Shut off all possible sources of ignition. Transfer to container and arrange for removal.
Waste Disposal:	Follow federal, provincial and local regulations; see Section B.2.
Handling Procedures and Equipment:	Avoid generating dust. Follow routine safe handling procedures.
Storage Requirements:	Suitable labelled containers. Store in a cool, dry, well-ventilated area out of direct sunlight.
Special Shipping Instructions:	Follow all Transport of Dangerous Goods regulations.

Appendix C

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Appendix D

Literature Sources

On-line database searches were conducted on the DIALOG system, including the following databases:

Aquatic Science and Fisheries Abstracts

- Biosis Previews
- Current Contents
- Enviroline
- Environmental Bibliography
- NTIS (U.S. National Technical Information Service)
- Oceanic Abstracts
- Pollution Abstracts
- Waternet
- Water Resources Abstracts

The searches focussed on literature pertaining specifically to the use of chemicals as reference toxicants since there was neither time nor budget to conduct a complete literature search on each chemical. Review reports were available for most chemicals, however, and these provided most additional information.

Appendix E

Test Solution Preparation

The preparation of accurate and consistent test solutions is critical to consistent reference toxicant test performance. Studies that investigated test precision have shown that inconsistency in toxicant concentration preparation can be a considerable source of variability when the solutions are not analytically verified (Dorn *et al.*, 1987; Lemke, 1981; DeGraeve *et al.*, 1989). Several rules-of-thumb will enhance the accuracy of the test solution concentrations:

- The concentration of the reference toxicant in the initial stock solution should not exceed its water solubility.

- Avoid pipetting very small volumes (e.g., less than 1 mL).

- Avoid excessive dilution steps. Typically the exposure concentrations can be prepared within two or three dilutions, from the solid or pure chemical form to the final test solutions; every additional dilution introduces further variability.

- Use appropriately accurate measuring devices and vessels such as:

  - an analytical balance or a balance with accuracy to at least two decimal places greater than the quantity to be weighed (e.g., accurate to within one per cent or less);

  - volumetric flasks of an appropriate size for stock solution preparation (a 1-L flask is usually a convenient volume to minimize errors in calculation during dilution steps);

  - graduated cylinders of a size appropriate for the volume of solution to be measured (e.g., measure 100 mL in a 100-mL cylinder, not in a 1-L cylinder); and

  - accurate pipets such as measuring, volumetric or fixed-volume (e.g., Eppendorf) pipets.

- If a reference toxicant is to be used for more than one test type, prepare a single initial stock solution at a concentration that is suitable for all tests; make working stocks for each test type from the original stock.

- Whenever possible, work with round numbers, preferably factors of 10; it is easier to work with solutions of 10 mL or 1 L than those of 22 mL or 2.2 L; similarly, the weight of chemical measured out for the initial stock solution should be an easy-to-use number. The extra time involved in weighing out 1.00 g to within 0.005 units, for example, will be offset by improved accuracy and precision in the exposure solutions and, therefore, in the test results.

- For chemicals that are stable in solution, it may be possible to prepare a stock concentration that can be stored and used for a number of tests over time. Chemical stability can easily be confirmed by re-analyzing a sample of the stock solution periodically over time to ensure that the toxicant concentration has not changed.

- All glassware should be properly cleaned and rinsed with dilution water prior to use.

An example of test solution preparation best demonstrates the steps that are typical in beginning a reference toxicant test. The first example presented in Table E.1 pertains to any chemical that has demonstrated an LC_{50} of approximately 100 µg/L to the test organism. It could also be used for sublethal testing if the threshold sublethal effects were expected to occur at about 40 µg/L. The example is also intended for a test organism that is tested in a volume of 1 L or less (e.g., *Daphnia magna* or *Ceriodaphnia dubia* or fathead minnows) since the final test solution volume is 1 L. The exposure concentration series that was then selected was 0, 10, 20, 30, 50, 100 and 200 µg/L, achieving (more or less) a geometric series but maintaining concentrations that are easily prepared. The exposure solutions were completed within three steps.

The second example was prepared to demonstrate the preparation of large-volume test solutions (e.g., 20 L) such as may be used in rainbow trout tests. In this case, a small volume of the initial stock solution can be added directly to the test vessel dilution water. The additional volume of stock solution will represent an insignificant increase in the total exposure volume and the accuracy of the final concentration will not be compromised.

Exposure concentrations and test results for metal salts should be reported on the basis of the metal itself (e.g., mg Cu/L).

Table E.1 Examples of Test Solutions' Preparation Steps (see text)

Step	Description	Quantity	Substance	Volume of water	Resulting Concentration
Example 1: LC_{50} = 100 µg/L, test volume = 1 L or less					
1.	Initial Stock	1.00 g[a]	Pure Toxicant	1.0 L[b]	1 g/L or 1000 mg/1000 mL or 1 mg/mL
2.	Working Stock	10 mL[c]	Initial Stock	1.0 L[b]	10 mg/L or 10 000 µg/1000 mL or 10 µg/mL
3.	Test Exposure Solutions	20 mL[c]	Working Stock	1.0 L[b]	200 µg/L
		10 mL[d]	Working Stock	1.0 L	100 µg/L
		5 mL[d]	Working Stock	1.0 L	50 µg/L
		3 mL[d]	Working Stock	1.0 L	30 µg/L
		2 mL[d]	Working Stock	1.0 L	20 µg/L
		1 mL[d]	Working Stock	1.0 L	10 µg/L
Example 2: LC_{50} = 300 µg/L, test volume = 20 L					
1.	Initial Stock Preparation	1.00 g[a]	Pure Toxicant	1.0 L[b]	1 g/L or 1000 mg/1000 mL or 1 mg/mL
2.	Test Exposure Solutions	10 mL[c]	Initial Stock	20 L	10 mg/20 L or 0.5 mg/L or 500 µg/L
		6 mL[d]	Initial Stock	20 L	6 mg/20 L or 0.3 mg/L or 300 µg/L
		4 mL[d]	Initial Stock	20 L	4 mg/20 L or 0.2 mg/L or 200 µg/L
		2 mL[d]	Initial Stock	20 L	2 mg/20 L or 0.1 mg/L or 100 µg/L
		1 mL[d]	Initial Stock	20 L	1 mg/20 L or 0.05 mg/L or 50 µg/L

[a] weighed on analytical balance
[b] use volumetric flask
[c] use volumetric pipette
[d] use fixed volume pipette, volumetric pipette or measuring pipette